Author:
O'Reilly, Liam J

Title:
Passive anti-bat defences in nocturnal Lepidoptera

novel wingbeat-powered sound production in deaf microlepidoptera, and acoustic crypsis in moths

Passive anti-bat defences in nocturnal Lepidoptera: novel wingbeat-powered sound production in deaf microlepidoptera, and acoustic crypsis in moths

Liam Joseph O'Reilly

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree Doctor of Philosophy in the Faculty of Life Science October 2019

Word count: 31,343

ABSTRACT

Nocturnal moths and echolocating bats have been in a co-evolutionary arms race for 65 million years. Resulting adaptations are complex, diverse, and often convergently evolved. Notable examples include anti-bat hearing providing an early warning of approaching predators, sound production to startle, warn, or jam an approaching bat, and potential acoustic camouflage afforded by the wing scales and body fur of nocturnal moths. Here, I investigate two aspects of passive anti-bat defence, wingbeat-powered anti-bat sound and acoustic camouflage.

I present the first combined description and acoustic characterisation of the novel wing-embedded sound-producing structure in the microlepidopteran genus *Yponomeuta*, which I term the aeroelastic tymbal (AT). By investigating the life history of *Yponomeuta* and comparing their acoustic characteristics with those of moth sounds in the literature, I conclude that they are acoustic Müllerian mimics of the chemically defended Arctiinae. I then show that ATs likely convergently evolved 15 times in the microlepidoptera, confirming sound production from five (including *Yponomeuta*) of the 15 examples. Due to their acoustic characteristics being similar to known anti-bat sounds and the life histories of some taxa placing them at high risk of bat predation, I conclude that these ATs too produce anti-bat sounds.

Research into potential acoustic camouflage in moths has previously focussed on small numbers of species, so I present the first species-rich, phylogenetically widespread study on the topic. Using acoustic tomography, I compare the ultrasonic retroreflection of nocturnal moths and two groups of diurnal Lepidoptera, butterflies and diurnal moths. Unlike previous work I find no differences between moths based on their diel (day-night) activity; however, moths as a whole show reduced echo intensity over all the tested frequencies for both their bodies and wings when compared to butterflies. It appears that moths are adapted for broadband acoustic camouflage against bats; whereas, butterflies are not.

DEDICATION

In memory of Matthew O’Reilly

ACKNOWLEDGEMENTS

Firstly, I thank my supervisor Dr Marc Holderied, for his incredible support, guidance, and advice, but also for giving me this opportunity and believing in me. I have learnt so much from him and without his supervision I would not have achieved what I have.

I must also thank Dr David Agassiz greatly. Since our chance meeting at the Bristol and District Moth Group AGM, we have made discovery after discovery. David's advice and specimen donation has been integral to my work, and without him I would not have delved into the wonderful world of microlepidopteran acoustic research.

Thank you to every member of the BASELab, you made working not feel like working, maybe because sometimes we weren't (perhaps you should get new oscilloscope that doesn't have a Tetris mode, Marc). Particular thanks go to Tom, Caitlin, Liam, and Dan for their moral support in my (happy) hours of need. All jesting aside, I have made great friends during my time with the BASELab, and that is in no short part down to the positive environment created by Marc and his lab members.

Thank you to the Coffee and Crossword Crewe, I always looked forward to 11am.

A special thank you goes to Nia; throughout this process you've made the rough times great and the great times greater. I do wish you wouldn't kill my study species though, even if they might eat your clothes.

Thank you to my family for their unwavering support over the years through the good and the bad. Thanks Mum, for always being there with wholesome food and a pint when I needed it most, and Dan for being a fantastic brother and an even better friend.

I would also like to thank the University of Bristol Science Faculty for funding my project through the Graduate Teaching Assistant Scheme.

AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED:……

DATE:……

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AT Aeroelastic Tymbal

CA Calibration tone level (dB)

TS Test signal amplitude (mPa)

CS Calibration signal amplitude (mPa)

HT Hearing threshold (dB SPL)

δ distance (m)

δref Reference distance (m)

CSL Click source level (dB peSPL at δref)

FDA Frequency-dependent attenuation (dB m^{-1})

TS Spectral target strength of moth echo (dB SPL at δref)

BSL Source level of bat call (dB SPL at δref)

TAT *Tinea*-like aeroelastic tymbal

MAT *Monopis*-like aeroelastic tymbal

TrAT *Trichophaga*-like aeroelastic tymbal

Chapter 1: General Introduction

Predator-prey interactions are a key element of community ecology. Where predators exist adaptations in the prey arise that increase their survivability, and counteradaptations subsequently evolve in the predators. This evolutionary back-and-forth is known as an evolutionary arms race. The key example is taken from the specialist acoustic nocturnal interactions of bats and nocturnal flying insects (e.g. Goerlitz *et al.*, 2010; Hofstede and Ratcliffe, 2016).

1.1 The bat-moth co-evolutionary arms race

The majority of Lepidoptera (Rhapalocera - butterflies and Heterocera - moths) are nocturnal moths (Kawahara et al., 2017), and the majority of bats are nocturnal insectivores specialising in hawking (hunting prey on the wing) flying prey (Altringham, 2011) with diets often consisting of considerable amounts of Lepidoptera (e.g. Dodd and Lacki, 2007; Clare *et al.*, 2009; Goerlitz *et al.*, 2010; Dodd *et al.*, 2012). As a result, the predator-prey relationship between nocturnal Lepidoptera (moths) and bats is intense and considered a textbook example of a co-evolutionary arms race due to the many resulting adaptations.

Lepidoptera began as a diurnal order followed by a switch to nocturnality (Kawahara et al., 2017). In fact, the Ditrysia (a natural clade consisting of around 98% of all extant Lepidoptera) are predominantly nocturnal. (Kawahara et al., 2017; Van Nieukerken et al., 2011). Kawahara et al.'s (2017) analysis revealed at least 49 subsequent evolutionary shifts from nocturnal to diurnal activity including in the butterfly superfamily Papilionoidea (the ancestral butterfly was likely diurnal); yet, the vast majority of Lepidoptera (75-85%) remain nocturnal (Espeland et al., 2018; Kawahara et al., 2017). The evolution of flight and echolocation in bats allowed them to exploit this huge number of nocturnal flying insects, and nocturnal Lepidoptera and bats have been locked in an evolutionary battle since. Moths are one of the most diverse groups of insects, containing 137,124 documented species (Van Nieukerken et al., 2011), and they have evolved a plethora of anti-bat defences, including many examples of convergent evolution. In fact, subsequent switches to diurnal activity in Lepidoptera may have occurred to avoid bat predation. Here I will focus on three defensive adaptations, hearing, sound production, and prey echo manipulation.

1.1.1 Hearing

The most well-known and widespread defence against bats is audition: hearing structures that can detect approaching echolocating bats (Conner and Corcoran, 2012). This defence is so successful that it is not only present in nocturnal Lepidoptera, but also four other insect orders

containing nocturnal flying species (Orthoptera, Coleoptera, Mantodea, and Neuroptera) (see Miller and Surlykke, 2001). Although anti-bat hearing has been investigated since the 1950s (Roeder and Treat, 1957), new examples of anti-bat hearing are still being found in nocturnal insects (e.g. Holderied, Thomas and Korine, 2018), and many more taxa likely possess this ability.

Anti-bat hearing tends to be achieved through structures known as tympanal organ, membranes connected to sensory neurons usually on the body of the insect, which are excited by the pressure changes of airborne sound waves (Hoy and Robert, 1996); although, the Sphingidae (hawkmoths) are a notable exception, their ears are modified mouthparts, not tympanal organs (Göpfert and Wasserthal, 1999). Anti-bat hearing is such a widespread defence in insects because they possess sensory organs (proprioceptors) predisposed to become hearing structures. Proprioceptors are specialised mechanoreceptors, which are used to monitor mechanical movement of the animal itself (Pringle, 1937). These structures appear to represent the origins of tympanic hearing structures; in atympanate relatives of eared insects the same nerves that innervate the tympanum are found to innervate proprioceptors, probably indicating that this was the ancestral state of these taxa (see Fullard and Yack, 1993).

Typanal hearing, whether for communication, predator detection, or host localisation by parasitic taxa, has been well-studied in seven insect orders (Hoy and Robert, 1996), but variation in the hearing structures themselves varies even within these orders. For example, the well-studied Noctuidae (Lepdidoptera) tympanal organ is innervated by two sensory neurons (A1 and A2) each with different detection thresholds (Roeder, 1964), yet the sister family to the noctuids, the Notodontidae, possess different innervation of their tympana with only one sensory neuron innervating the structure (Surlykke, 1984). A1 cells have been found to be 20 dB more sensitive than A2 and this difference in sensitivity may be linked to the behaviours associated with detecting an approaching bat (Roeder, 1964).

Once an insect detects an approaching bat it can defend itself in several ways, the most common and classic response is to perform stereotypical aerobatic escape manoeuvres (Conner and Corcoran, 2012). These behaviours vary depending on the perceived risk of the threat from the approaching bat, and interestingly could be linked, in the noctuids, to the different firing thresholds of their A1 and A2 cells, with activation of just A1 perhaps indicating a bat is distant, but firing of both indicates the threat is closer (Roeder, 1964). If the bat is perceived to be far away (low intensity sound detected) and thus predation risk is low, then the insect will change

its flight course to avoid detection. If the bat is perceived to be closer (high intensity sound detected), the insect will perform more drastic manoeuvres, for example, flight cessation (sudden landing) and diving from the air (Conner and Corcoran, 2012)

In addition to defensive manoeuvres other insects exhibit different behaviours to defend themselves upon detecting a hunting bat. Some male moths and Orthoptera will cease acoustic sexual displays so as not to draw attention to themselves (Spangler, 1984), and some moths and tiger beetles will reply with sounds of their own (Conner and Corcoran, 2012; Yager and Spangler, 1997).

1.1.2 Anti-bat sound production

Sound production following the detection (by acoustic or tactile stimulation) of a hunting bat is an effective anti-bat strategy (Conner and Corcoran, 2012). Anti-bat sound production has been studied for decades in the Arctiinae (Erebidae, tiger moths) (e.g. Surlykke and Miller, 1985; Simmons and Conner, 1996; Barber and Conner, 2007; Dowdy and Conner, 2016), these chemically defended moths are often visually aposematic and, in order to convey aposematic signals to bats in the absence of light, they also use conspicuous acoustic signals (Hristov and Conner, 2005a). Such signals manifest themselves as ultrasonic clicks produced by structures known as tymbals

1.1.2.1 Tymbal organs

The majority of moth anti-bat sound production is achieved through tymbal organs found on various regions of the insects; from the abdomen, and thorax to the wings (Blest et al., 1963; O'Reilly et al., 2019; Skals and Surlykke, 1999). However, tymbals are not exclusive to the Lepidoptera, and are perhaps most well-known as the sound production mechanism of male cicadas (Hemiptera). Generally, a tymbal consists of a thin, stiff region of cuticle (a membrane) backed by an air cavity and connected to a muscle. Simply put, contraction of the muscle actuates the tymbal by buckling the membrane which produces a click, relaxation of the muscle then returns the structure to its resting state, again producing a click. Sounds are amplified by the air cavity behind the membrane acting as a resonator (Fullard and Heller, 1990). In the case of cicadas, and indeed many moths, tymbal sound production has an added layer of complexity, multiple click production per buckling event, i.e. muscle contraction. Cicadas possess ribs which each buckle in sequence following initial tymbal actuation producing a train of clicks, and something similar is achieved by moths using tymbal

striations known as microtymbals (Fenton and Roeder, 1974; Young and Bennet-Clark, 1995).

It is not just the Arctiinae that produce anti-bat sounds, the defence mechanism has evolved convergently in multiple moth families, and even in the Coleoptera (beetles). At least one species of geometrid uses independently evolved thoracic tymbals (Corcoran and Hristov, 2014), some sphingids produce sound using genital structures (Barber and Kawahara, 2013; Kawahara and Barber, 2015), the microlepidopteran genus *Yponomeuta* (Yponomeutidae) uses novel wingbeat-powered tymbals (O'Reilly *et al.*, 2019, see Chapter 2), and some tiger beetles respond to bat echolocation calls with ultrasonic clicks probably produced by their elytra/wings (Yager and Spangler, 1997). These tiger beetle species (e.g. *Cicindela marutha*) possess hearing structures on the dorsal surface of their abdomen, below their elytra (wing cases) and respond to a broad range of ultrasound relevant to bat echolocation calls. In addition to producing trains of ultrasonic clicks, the beetles have a stereotypical response, kicking their legs, rolling their head, swinging their elytra and increasing their wingbeat frequency and excursion, upon detecting bat-like sounds.

1.1.2.2 Function: startle, jamming, and aposematism

Anti-bat sounds can function in three not necessarily mutually exclusive ways; startling bats, jamming their biosonar, and providing them with an aposematic signal (Conner and Corcoran, 2012). Startle could function by eliciting the mammalian startle response in naïve bats. The mammalian startle response is a reflex seen in almost all mammals, coordinating hundreds of simultaneous muscle contractions in response to sudden, high intensity stimuli. Typically, the startled animal closes its eyes and hunches preparing it for fight or flight (Sillar et al., 2016). In this discussed predator-prey interaction, a bat's attack would be interrupted by this reflex in response to a moth's acoustic signal, allowing the moth additional time to escape. Startle may work if sound producing moths are rare, but as mammals, bats are quick to learn and may habituate to signals they encounter regularly, and although response latency can be subject to sensitisation in some mammals experiencing high intensity sound (Götz and Janik, 2011), there is direct evidence for startle function being ephemeral in bat-moth interactions (see Bates and Fenton, 1990; Hristov and Conner, 2005b). Therefore, it is unlikely that moths can gain protection solely from their clicks startling bats, and more enduring protection could be provided through aposematism or jamming.

Jamming appears to be rare and has only been proven through tests with bats in three moth species, one arctiine (*Bertholdia trigona*) and two sphingids (*Xylophanes tersa* and *X. falco*) (Corcoran et al., 2009; Kawahara and Barber, 2015). Acoustic characteristics of sounds can be used to determine whether an anti-bat sound could jam biosonar as these sounds require a duty cycle of at least 20% (Conner and Corcoran, 2012; Corcoran et al., 2010). Due to their clicks having a duty cycle greater than 20%, other Arctiinae species have been proposed as sonar jammers from analysis of their anti-bat sounds (Corcoran et al., 2010). When analysing anti-bat sounds duty cycle can be used to rule out jamming as a possible function. Three hypotheses for the mechanism of anti-bat sonar jamming have been put forward, the phantom echo hypothesis, the ranging interference hypothesis, and the masking hypothesis. Respectively they can be defined as, moth clicks being interpreted as echoes by bats, moth clicks reducing the accuracy of a bats ability to determine target range, and moth clicks overshadowing the echoes of the moth rendering it invisible. Behavioural tests with bats and moths by Conner and colleagues provide evidence in support of the ranging interference hypothesis (Corcoran et al., 2011).

Acoustic aposematism is the most common function of anti-bat sounds. As bats cannot utilise vision to detect prey and assess their profitability at night, unpalatable prey must inform them of their secondary defences through a different sensory modality, acoustics. Bats are quick to learn to associate salient ultrasonic clicks with unprofitable prey and post-exposure they will avoid clicking prey (Barber and Conner, 2007). Salient sounds therefore function as analogues of conspicuous visual warning signals such as bright colours.

As with the visual modality, acoustic aposematism in communities involves mimicry rings. These are groups of prey that exhibit the same aposematic signal but vary in the quality/presence of the secondary defence it advertises. Some mimics may possess a potent secondary defence such as toxins and are known as Müllerian mimics, whereas, others will be palatable Batesian (imposter) mimics. In the mid-2000s both types of mimicry were verified in the acoustic modality (Barber and Conner, 2007) and examples of both types of mimic are known for anti-bat sounds. For example, the palatable geometrid *Eubaphe unicolor* is an acoustic Batesian mimic of Arctiinae, and as many Arctiinae are toxic they represent examples of Müllerian mimics (Boppré, 1990; Corcoran et al., 2010; Corcoran and Hristov, 2014).

Despite anti-bat sound production being known solely from the Arctiinae for several decades, recent discoveries are demonstrating its widespread, convergently evolved presence in

nocturnal insects (Yager and Spangler, 1997; Barber and Kawahara, 2013; Corcoran and Hristov, 2014; Kawahara and Barber, 2015; O'Reilly *et al.*, 2019, see Chapter 2). The continued discovery of anti-bat hearing and sound production indicates that these defences are likely to be prevalent in other nocturnal insects and, thus, require further investigation. Focus should fall on the microlepidoptera as there is a dearth of research regarding anti-bat defences in this group despite bat diet analyses suggesting they are under significant predation pressure from certain bat species (e.g. Dodd *et al.*, 2012).

1.1.3 Echo manipulation

As many bats rely on echolocation for prey detection, manipulating their echo can be an effective defence for insects against bats. In fact, echo manipulation as a means of avoiding bat predation is becoming an increasingly well-studied area of the bat-moth arms race. Examples are the hindwing tails of some Saturniidae (silkmoths), which act as acoustic decoys guiding bat attacks away from the body towards the less important wing extremities (Barber et al., 2015; Lee and Moss, 2016), the possibility that moths avoid resting on smooth surfaces to reduce their "acoustic shadow" (Clare and Holderied, 2015), the potential acoustic absorptive power of moth wing scales and body fur (acoustic camouflage) (Neil et al., 2018; Ntelezos et al., 2017; Shen et al., 2018; Zeng et al., 2011), and even *Hepialus humuli* (Hepialidae), which fly close to vegetation to mask their echo against those of the surrounding clutter (Rydell, 1998).

The first investigation into this topic was in the 1960s, looking at the difference in the echo strength of wings with and without wing scales. However, despite confirming that scales reduce echo intensity mildly, the results were initially dismissed as insignificant (Roeder, 1962). As technology has improved, it has permitted further study of this concept; microreverberation chambers have allowed for the measurement of sound absorption of moth body parts (Ntelezos et al., 2017; Zeng et al., 2011), and the invention of novel ultrasonic acoustic tomography allows spectral and angular measurements of whole moth echoes as well their constituent parts replicating bat biosonar (Clare and Holderied, 2015; Neil et al., 2018). Additionally, laser Doppler vibrometry can measure the resonance properties of microscopic objects and has even been used to quantify the resonances of a single moth scale. This study demonstrated that the resonances of this scale match the frequencies of bat echolocation calls, and the potential absorptive power this confers matches the absorptive power of moth wings measured in the literature (Shen et al., 2018).

Studies of acoustic camouflage thus far have shown that moth wing scales absorb more ultrasound than those of butterflies (Zeng et al., 2011), nocturnal moths' wings absorb more ultrasound than diurnal species even showing intraspecies differences due to sexual differences in diel (day-night) activity (Ntelezos et al., 2017), and that the thoracic fur of moths confers broadband reductions in echo strength compared to butterflies (Neil et al., 2018). However, all these studies have investigated low numbers of species; Zeng *et al.* two moths and two butterflies, Ntelezos *et al.* six diurnal moths and three nocturnal moths, and Neil *et al.* two moths and two butterflies. Clearly, these differences could be species specific and consequential of small species sample sizes. Therefore, further study is needed with a wide range of species of butterflies, nocturnal and diurnal moths to determine whether the differences seen in these studies are consistently found across a phylogenetically spread data set.

1.2 Chapter outlines

I present three data chapters here investigating two aspects of passive anti-bat defences in moths. The first chapter addresses the bioacoustics of a striated microlepidopteran wing structure thought to produce sound but awaiting acoustic investigation (Agassiz, 2017). The second explores the phylogenetic spread of this structure within the lepidopteran suborder microlepidoptera. The third and final data chapter presents a phylogenetically widespread investigation into the echo strengths of Lepidoptera of varying diel activities, and thus predation threat from bats (butterflies, diurnal moths, and nocturnal moths).

Chapter 2 is an adaptation of an article published in Scientific Reports (O'Reilly et al., 2019). The aims of this chapter were to 1. determine whether the structure discovered in the wings of *Yponomeuta* moths by Agassiz (2017) is a sound producing structure, 2. characterise any sounds these moths produce, 3. determine how the structure functions to produce any sounds, and 4. suggest a function of any sounds, and 5. propose a biomechanical mechanism of any sound production. I address these aims by combining behavioural and acoustic tests with state-of-the-art acoustic tomography and high-speed videography.

Through Chapter 3 I addressed two aims. 1. to thoroughly explore the phylogenetic spread of structures similar to those of *Yponomeuta* within the microlepidoptera, and 2. using live moths available to me, test candidate structures for acoustic functionality, as well as characterise any resulting sounds. I achieved this through morphological examination of museum specimens as

well as online images of specimens, mapping the results to the latest molecular phylogenies, and behavioural and acoustic tests of locally available microlepidoptera.

Chapter 4 investigates the phenomenon of acoustic camouflage in moths. Previous studies on the topic show that nocturnal moths appear to absorb more ultrasound than butterflies and diurnal moths, or that their echoes are reduced by the presence of scales or hair (Neil et al., 2018; Ntelezos et al., 2017; Zeng et al., 2011). There is still a distinct lack of a species-rich, phylogenetically spread analysis of this phenomenon and this is what I address in Chapter 4. I aimed to assess the spectral echo strengths of a wide range of butterflies, diurnal moths and nocturnal moths by measuring their retroreflection of ultrasound at a range of frequencies relevant to bat echolocation (20-100 kHz, covering the majority of bat species). I used acoustic tomography to measure the echo intensities of 45 butterfly species, 41 diurnal moth species, and 48 nocturnal moth species spread over multiple families for each group.

Chapter 2: Acoustic Müllerian Mimicry in a Deaf Moth: Acoustic Characterisation and Proposed Function of *Yponomeuta* (Yponomeutidae: Lepidoptera) Sounds*

The data for Figure 2.11 were gathered by myself and Dr Thomas Neil, and the figure itself was created by Dr Neil under my instruction.

*This chapter incorporates material from the published manuscript "Deaf moths employ acoustic Müllerian mimicry against bats using wingbeat-powered tymbals", Sci. Rep. 9. 1444. 1-9. See author contributions for the paper below.

I and Dr Marc Holderied conceived the study. I led the data collection for the study and Dr Thomas Neil also participated in behavioural, and acoustic tomography data collection. I and Dr Holderied shared writing and I led data analysis. Dr Holderied offered advice and MATLAB code for acoustic tomography analysis, and Dr Neil participated in acoustic tomography analysis. Dr David Agassiz provided expert advice on Yponomeutidae as well as live specimens for acoustic and video recordings, and dead specimens for SEM data.

2.1 Abstract

Emitting ultrasound upon hearing an attacking bat is an effective defence strategy used by several moth taxa. Here I reveal how Yponomeuta moths acquire sophisticated acoustic protection despite being deaf themselves and hence unable to respond to bat attacks. Instead, flying Yponomeuta produce bursts of ultrasonic clicks perpetually; a striated patch in their hindwing clicks as the beating wing rotates and bends. The structure is strikingly similar to the thoracic tymbals with which arctiine moths produce their well-studied anti-bat sounds. And indeed, Yponomeuta sounds closely mimic such arctiine signals, revealing convergence in form and function. Because both moth taxa contain noxious compounds, I conclude they are mutual Müllerian acoustic mimics. Yponomeuta's perpetual clicking would however also attract insectivorous bats. In response, their click amplitude appears adapted to afford acoustic protection just as far as required; detectability of their clicks to bats matches the distance over which bat biosonar would pick up Yponomeuta echoes anyway – advanced acoustic defences for a deaf moth.

2.2 Introduction

Sound plays an important role in the life histories of many Lepidoptera, particularly for nocturnal moths. The lack of light during the scotophase prevents visual communication, and the principal nocturnal predators of these insects are echolocating bats, auditory specialists. Both the production and reception of sound play important roles in communication and predator defence in many moths. Audition has evolved independently at least 10 times in the Lepidoptera (Greenfield, 2016), and is present in almost half the order (Conner and Corcoran, 2012; Ratcliffe, 2009; Skals and Surlykke, 1999). It is widely accepted that hearing in nocturnal moths has evolved as a defence against echolocating bats (Fullard, 1988; Ratcliffe, 2009), providing these insects with an early warning of the approaching predators and allowing them to defend themselves through aerobatic manoeuvres and/or sound production (Miller and Surlykke, 2001). Interestingly though, research into the anti-bat sounds of hawkmoths (Sphingidae) has raised the possibility that the evolution of the hearing structures in these moths may have been influenced by mating as well as, or even initially instead of, bat predation pressure (Barber and Kawahara, 2013; Kawahara and Barber, 2015).

2.2.1 The bat-moth evolutionary arms race

Bats and moths have been involved in a 65-million-year evolutionary arms race since the advent of biosonar in Chiroptera (Conner and Corcoran, 2012). As a result, moths have evolved

a plethora of defences against their chiropteran adversaries. Some possess hearing structures tuned to the echolocation frequencies of sympatric bats (Fullard, 1994; ter Hofstede et al., 2013) providing an early warning system and allowing time for evasive manoeuvres. Others have long hindwing streamers that divert the attacks of bats are present in several large genera (Barber et al., 2015), and sound production as a defence against bats has evolved independently in at least three moth families (Barber and Kawahara, 2013; Corcoran et al., 2010; Corcoran and Hristov, 2014). Many bat species detect and localise prey by the sounds they generate (Fenton et al., 1983; Holderied et al., 2011), so sound production is only adaptive when it creates protection with the sounds startling attacking bats, warning them of a (chemical) defence or jamming their biosonar (Corcoran et al., 2011, 2009; Hristov and Conner, 2005b).

2.2.2 Acoustic communication in Lepidoptera

The ability of Lepidoptera to use hearing and sound production subsequently resulted in the evolution of acoustic communication in multiple lineages (Greenfield, 2014) as a method other than chemical or visual cues to facilitate mating and territory defence (Alcock and Bailey, 1995; Monge-Nájera et al., 1998; Nakano et al., 2013). Examples of acoustic communication are both plentiful and diverse. These range from the extremely quiet courtship sounds of the males of many eared moth species, which are inconspicuous to unintended targets (Nakano et al., 2009), to the long-distance communication signals of male *Hecatesia* species (Noctuidae) produced by forewing 'castanets' (Alcock and Bailey, 1995).

2.2.3 Mechanisms of sound production in Lepidoptera, with particular focus on tymbals

Lepidoptera produce sounds in a plethora of ways, but when broken down to their base components these sounds almost all consist of very short broadband clicks. The spectral components and temporal composition of these clicks differs and thus provides the variation between taxa. Clicks can be produced in a number of ways, through scraping surfaces against each other such as the scraping of specialised scales (Barber and Kawahara, 2013; Nakano et al., 2008) or stridulation (Gwynne & Edwards 1986; Surlykke & Gogala 1986; Lees 1992), or through various forms of tymbals (Corcoran and Hristov, 2014; Dowdy and Conner, 2019; Fenton and Roeder, 1974; Heller and Achmann, 1993; Jang and Greenfield, 1996; Skals and Surlykke, 1999), thin areas of cuticle, often consisting of a smooth area adjacent to a striated band, backed by an air cavity, which are normally buckled in and out by a dedicated muscle (Blest et al., 1963). Tymbals are not exclusive to the Lepidoptera and are also found in the Hemiptera, specifically in the cicadas, where, like those of the Lepidoptera, these tymbals produce sounds through buckling (Young and Bennet-Clark, 1995). However, whilst many

lepidopteran tymbals, particularly those of the tigermoths, are used by both sexes to produce sounds for defence against bats (Corcoran et al., 2010), cicada tymbals are exclusively used by males to produce their loud courtship songs (Young and Bennet-Clark, 1995).

There is great diversity in the placement of tymbals in the Lepidoptera; thoracic, abdominal, tegula-based, and wing-based tymbals have evolved in nocturnal moths and all function to produce sound for either communication or defence (Blest et al., 1963; Heller and Achmann, 1993; Jang and Greenfield, 1996; Skals and Surlykke, 1999). Despite these differences in placement on the insect, tymbals all produce sound through buckling. Thin areas of cuticle buckle (snap) through, which produces a very short sound impulse, a click. Moth clicks are often produced in bursts, which can be created by repetitive actuation of the tymbal buckling and/or through a series of independent microtymbals, which appear as striations on the tymbal. High-speed videography of buckling arctiine tymbals has shown that when the tymbal is buckled by a muscle contraction the microtymbals buckle in succession each producing a click (Corcoran and Hristov, 2014). Relaxation of the muscle results in the same buckling but in the opposite direction, returning the tymbal to its resting state and again producing a click. The result of one full contraction and relaxation cycle of the muscle is two trains or bursts of ultrasonic clicks (two combined bursts/tymbal buckling events is known as one modulation cycle) (Blest et al., 1963; Corcoran and Hristov, 2014) (Figure 2.1).

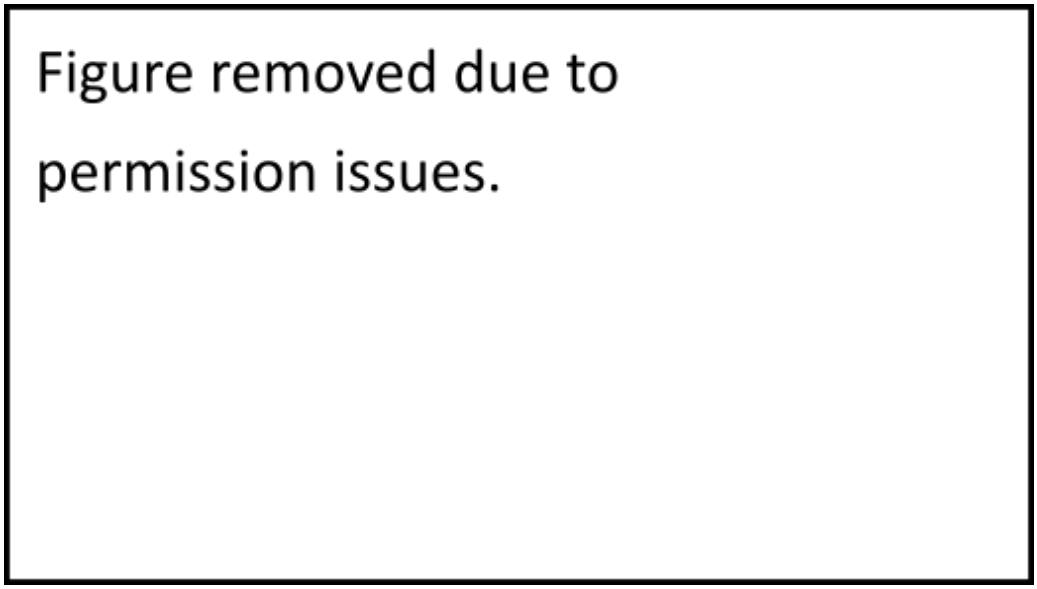


Figure 2.1 Tigermoth tymbal Stills from high-speed video of *Cycnia tenera* tymbal actuation. Stills show one full modulation cycle (buckling in and out of the tymbal). Microtymbals are circled in white and buckling can be seen progressing from the top of the band of microtymbals in plots A-C which correspond to the first burst of clicks in the time signal (marked in red). The buckling of the microtymbals as they return to their resting state can be seen in plots D-E and corresponds to the second click burst in the time signal (marked in blue). The stills and audio were modified from supplementary material from Corcoran and Hristov (2014).

In addition to the widespread evolution of tymbals, convergence in lepidopteran sound production can also be seen through the prevalence of acoustic structures found on the wing. Male *Amyna natalis* possess a tymbal embedded in the forewing to produce communication sounds, but there are additionallynumerous wing-based sound producing structures used for communication. For example the Australian whistling moth (*Hecatesia* spp., Noctuidae) uses percussive castanets (large swellings) on its forewings to produce territorial acoustic emissions (Alcock and Bailey, 1995) and *Hamadryas* and *Heliconius* butterflies (both Nymphalidae) use, as yet unelucidated, wing-based sound production mechanisms for intra, and in the case of *Heliconius*, interspecific communication (Hay-Roe and Mankin, 2004; Yack et al., 2000).

2.2.4 Yponomeuta

Yponomeuta Latreille, [1796] (Lepidoptera; Yponomeutidae) is a genus of likely over 100 species (Agassiz, D. Personal Communication, Mar 2018) of small ('microlepidoptera') to medium sized, mostly nocturnal moths (Heppner, 2008; Turner et al., 2010). The genus is widespread in the Palearctic region, and one species is present in North America (Turner et al., 2010). There is a vast amount of literature covering *Yponomeuta* species as they are used as models for the evolution of the relationship between host plants and phytophagous insects (e.g. Menken et al. 1992; Roessingh et al. 2000). Morphologically, the genus has been characterised by the presence of a hyaline patch devoid of scales at the hindwing base, between Cu_{1b} and Cu_2 veins (Figure 2.2) (Meyrick, 1895). Such a patch is also known from related genera of the subfamily Yponomeutinae, *Teinoptila*, *Ptiloteina*, *Trisophista*, *Eumonopyta* (Agassiz, 2017; Sohn, 2016).

Agassiz (2017) found that these hyaline patches contain a row of ridges adjacent to the Cu_2 vein (Figure 2.2b), and proposed sound production as their function by stridulation, naming the structure itself a *stridularium*. However, the structure also resembles the thoracic tymbals of arctiines.

In fact, one observation exists of *Yponomeuta evonymella* and *Y. padella* producing ultrasound during flight in the field (Ahlen, 1997). The nocturnal activity of *Yponomeuta* means any sound production, regardless of the intended function, will have an influence on the relationship of the insect with bats. Very little is known about the evolutionary (acoustic) arms race between bats and the 'microlepidoptera', let alone *Yponomeuta* specifically (Gonsalves et al., 2013; Waters et al., 1995).

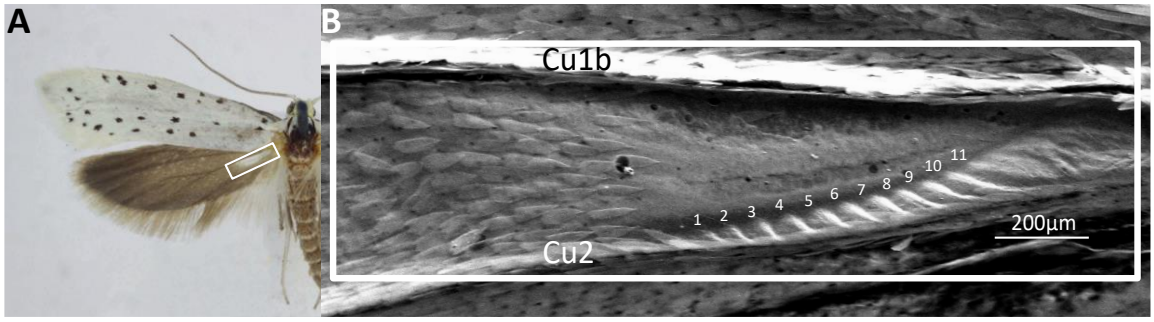


Figure 2.2 Hyaline (translucent) patch of *Yponomeuta* (A) *Yponomeuta evonymella* exposing the hyaline patch (in white box; see B) on its hindwing. (B) Scanning electron micrograph (SEM) of the ventral side of the hyaline patch (white box corresponds to that in A), with the striations numbered from left to right and the Cu_{1b} and Cu_2 veins labelled.

2.2.5 Aims

Through this project I aimed to 1. determine whether the hyaline patches of *Yponomeuta* and, by association, their relatives are indeed sound producing structures, 2. characterise the acoustics of any sounds these moths produced, in terms of temporal, amplitude and spectral information, 3. propose a function of any sounds produced by these moths, 4. compare detection distances by echolocating bats (a likely predator of these moths) of any sounds these moths produce with the detection distances of the moths based on their biosonar footprint, and 5. propose a biomechanical mechanism by which the hyaline patch may produce sound.

Given the appearance of the hyaline patches of *Yponomeuta* and their relatives, and their similarity, in terms of being formed of a striated band bordered by a scaleless flat region, to arctiine tymbals (Agassiz, 2017; Fullard and Heller, 1990), I predicted that it was indeed a sound producing structure. Again, due to the structural similarities with arctiine tymbals, I predicted that the sounds *Yponomeuta* produced would be akin to the anti-bat sounds of the Arctiinae, i.e. bursts of broadband ultrasonic clicks (Corcoran et al., 2010). The previous prediction also led me to predict that any sounds these moths make would be anti-bat sounds, and that these sounds would therefore need to be detectable by bats before they located the moth using biosonar, in order to function successfully before the moth is predated. It is also possible that these sounds could be intraspecific communication signals; however, as no hearing structures have been discovered in the Yponomeutoidea superfamily this seems less likely (Agassiz, 2017), therefore I also predicted that *Yponomeuta* would not respond to ultrasonic signals. Due to the position of the structure on the wing membrane, with no obvious direct muscle attachment I predicted that wing movement during flight would be responsible for actuating the structure.

2.3 Methods

The experiments detailed here were either conducted between July and August in 2017 or 2018.

2.3.1 Insect selection and collection

Yponomeutinae were chosen based on availability; live specimens of three British *Yponomeuta* species (Yponomeutidae, Lepidoptera), *Y. evonymella*, *Y. cagnagella*, and *Y. padella* were used during the investigation. All specimens were wild caught as larvae and reared to pupation. All *Y. evonymella,* four *Y. cagnagella* and the one *Y. padella* individual were provided by donors, all other *Y. cagnagella* were collected and raised by me. Pupae were kept in the lab until eclosion within 297x159x102 mm plastic rearing boxes (WorldwideButterflies, Lulworth, United Kingdom) at 21°C. Ablation experiments were performed between July and August 2017 using *Y. evonymella*. Directionality experiments were performed on *Y. evonymella* between July and August 2017 and *Y. cagnagella* and *Y. padella* in July 2018. Limited numbers of individuals during 2017 meant that they were used for both ablation and directionality experiments, therefore recordings were made from only the right-hand-side of the insects as they became exhausted and recordings could not be made from 360°. As there was no need to perform ablation experiments in 2018 recordings were made from 360° on *Y. cagnagella* and *Y. padella*.

2.3.2 Tethering method

Due to their small size, standard methods of tethering such as adhesives failed, so a size 000 insect pin was inserted dorsally into the mesothorax/prothorax of the moth until it just protruded ventrally. Although this is obviously an invasive tethering method, tethered specimens continued to fly for prolonged periods of time. Both audio and video recordings showed no obvious difference in the sounds produced by moths or their behaviour between tethered and free flight, so I continued with this as my tethering method. To account for possible muscle damage, I intended to only use specimens which showed pre-tethering flight behaviour, but all individuals did, thus, there was no need to be selective with specimens.

During 2017, the pinhead was attached to a piece of dowel (5 mm in diameter) which itself was clamped so the moth was suspended in the centre of the flight arena. 2018 experiments were performed with the pinhead inserted into modelling clay attached to a flexible arm

(Manfrotto + Co. Spa, Cassola, Italy), and the moth was positioned upside down (Figure 2.3).

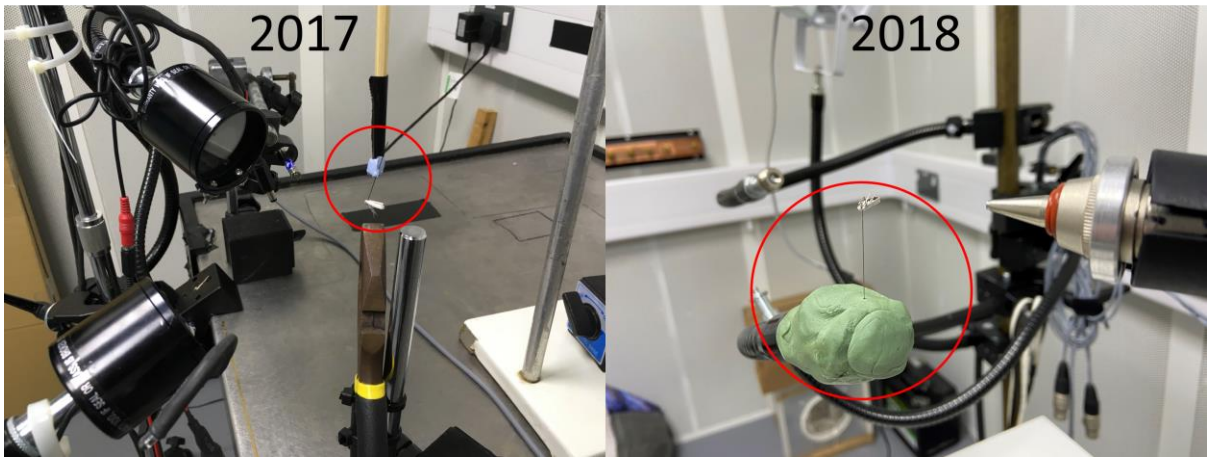


Figure 2.3 Different moth tethering methods Tethering methods in 2017 and 2018. The key difference being the orientation of the moth (upside down in 2018). Tethered moths are circled in red.

2.3.3 Ablation

Twelve *Yponomeuta evonymella* were tethered, flown, and recorded with their hindwings intact. The moths were positioned in the flight set-up and left, holding a small piece of foam to simulate being sat on a surface for 15-30 minutes or until they initiated flight themselves. If they had not initiated flight by then, it was elicited by removing the piece of foam they were holding, which reliably triggered flight.

Under a 50x magnification dissection microscope (Leica EZ5 Stereo Microscope, Leica Microsystems, Wetzlar, Germany) the hyaline patches in both hindwings were then removed using microdissection scissors from the wing joint to the point where scales began to appear. All ablated individuals were alive after that treatment and continued to fly on a tether with no noticeable difference in their flight pattern and readiness. Their sounds were then recorded again using the same procedure.

2.3.4 Audio and video recordings

All audio recordings were made within a semi-anechoic chamber (Industrial Acoustics Company Ltd., Winchester, UK) and recorded at 16bit with a sampling rate of 300 kHz.

2.3.4.1 2017

Audio recordings were made using a Type 4954 ¼“ free-field microphone (grid on) with a Type 2669-L preamplifier, connected to a Type 2690 NEXUS conditioning amplifier (all Brüel and Kjær Sound and Vibration Measurements A/S, Nærum, Denmark), run through National Instruments NI-USB-6251 BNC sound card (National Instruments, Austin, Texas, United States). The software used to make these recordings was AviSoft Ni-Daq Recorder (Avisoft

Bioacoustics, Berlin, Germany). See Appendix 1 for the frequency response of the microphone. The microphone was positioned between 70 and 135mm from the insect.

Insects were released or tethered within a 24x24x24" BugDorm-1 Insect Rearing Cage (Megaview Science Co., Ltd., Taichung City, Taiwan) lined on the base, back and one wall with ultrasound absorbing foam (Studiofoam 4" Pyramids, Auralex Acoustics Inc., Indianapolis, IN) to reduce echoes and reverberation. The recording microphone was positioned through a small circular hole cut into the mesh on the unlined side of the cage. The front panel (facing the camera) and the right-hand panel of the BugDorm-1 were removed for synchronous audio and video recordings in order to facilitate the activation of the synchronisation click.

Video recordings were made in the same set-up as above with the camera (Photron FASTCAM SA1.1, Photron, Tokyo, Japan) lens (Nikon Micro-NIKKOR 105mm prime lens, Nikon, Tokyo, Japan) positioned through a sleeve opening of the BugDorm-1 and pointing perpendicular to the microphone axis. Video recordings were made at 3000fps with a resolution of 1024x1024 pixels and the subject was illuminated using infrared (IR, 850 nm) lighting from four LED light sources.

Video and audio recordings were synchronised using a visual and acoustic signal analogous to a clapperboard. A pair of pliers which produced an intense (louder than all other acoustic signals in the set-up), rapid, broadband click was kept in frame and shut once during each recording session. The frame (video) and sample (audio) number containing the impact of the pliers jaws were easily discernible and these numbers were used to synchronise the audio and video files.

2.3.4.2 2018

Audio recordings were made using USG Omnidirectional Electret Ultrasound Knowles FG-O microphones connected to an UltraSoundGate 1216H^{200} recorder (both Avisoft Bioacoustics, Berlin, Germany). See Appendix 4 for the frequency response of the microphone. These recordings were made using Avisoft Recorder USGH (Avisoft Bioacsoutics, Berlin, Germany). The insect was positioned between two microphones facing one another in order to simultaneously record sounds from two orientations 180° apart. The microphones were positioned between 30 and 50mm from the insect but were always equidistant. Recordings were not made within the BugDorm-1 this year.

2.3.5 Hearing tests

Twenty *Y. evonymella*, and four *Y. cagnagella* were free flown in a semi-anechoic chamber with and without exposure to an ultrasonic stimulus known to elicit the anti-bat behaviours of insects possessing anti-bat hearing (Juliana et al., 2007). Two human observers documented the behaviour of each individual under both conditions. A reaction was defined as the sudden cessation of flight, any typical anti-bat escape manoeuvre, or change in flight direction. Each moth was flown twice and one observer chose the order of stimulus exposure for each individual, while the other observer was kept blind to the condition. A Dazer II Ultrasonic Dog Deterrent (Dazer International, London, UK) was used as the stimulus, between one and two metres from the subject. The Dazer II produces a 25 kHz tone at 118.1dB SPL (at 0.1 m), which is similar in frequency and intensity to bat echolocation calls (Bogdanowicz et al., 1999; Holderied et al., 2005), and is known to elicit anti-bat behaviour (Juliana et al., 2007). All 24 individuals (separated by species) were also ensonified at rest within a 24x24x24" BugDorm-1 Insect Rearing Cage (Megaview Science Co., Ltd., Taichung City, Taiwan), at a distance of around one metre from the centre of the cage.

2.3.6 Acoustic analysis

All sound recordings were analysed using Avisoft SASLab Pro (version 5.2.07, Avisoft Bioacoustics, Berlin, Germany). Through synchronised high-speed video and audio recordings of 10+ individuals it became apparent that wingbeats could be determined from the time signal of audio recordings. A set of two click bursts (one long and one short) represents one full wingbeat, with each burst representing one of the wing strokes (up or down). These sets of two click bursts were therefore used to determine a full wingbeat simply from the time signal of acoustic recordings. For each individual specimen, click bursts from ten consecutive wingbeats were analysed, counting all clicks or further analysing the loudest click from each upper, i.e. occurring during the upstroke click burst. Click number was determined by totalling the number of clicks discernible in waveform and spectrogram for each of the two click bursts per wingbeat (there are two click bursts per modulation/wingbeat cycle, upper and lower). Individual click duration was measured manually from the waveform. Click amplitude was calculated as peak-to-peak sound pressure values using the waveform of individual clicks, and was then converted to dB peSPL using a calibrated 40 kHz signal generator which produced a constant tone (Avisoft Bioacoustics, Berlin, Germany) and using the following formula:

$$CA + 20 * log_{10}\left(\frac{TS}{CS}\right)$$

$$CA = Calibration\ Tone\ Level\ (dB)$$

$$TS = Test\ Signal\ Amplitude\ (mPa)$$

$$CS = \ Calibration\ Signal\ Amplitude\ (mPa)$$

For spectral analysis, individual clicks were isolated from the waveform including a linear ramp of 0.05 ms of noise on either side. Silence was then added (zero padding) on either side before spectral analysis. Peak frequency was determined from a power spectrum (Hamming window size 1024). High and low frequencies (bandwidth) were calculated from the frequencies ±15dB below the amplitude of the peak frequency.

2.3.6.1 Detection Distance

Click detection distances were calculated from the loudest click from the upper burst of ten consecutive wingbeats. The peak frequencies and amplitudes (dB peSPL) of each click were used to calculate the distance at which these sounds could be detected by bats, using a bat hearing threshold of 10dB SPL. The following formula, an adaptation of the sonar equation (Møhl, 1988), was used to calculate these distances.

$$CSL - 20 * log_{10}\left(\frac{\delta - \delta ref}{\delta ref}\right) - FDA * (\delta - \delta ref) = HT$$

$$HT = \text{hearing threshold} = 10\ \text{dB SPL}$$

$$CSL = \text{Click Source Level (dB peSPL at } \delta\text{ref)}$$

$$\delta = \text{Distance (m)}$$

$$\delta ref = \text{Reference Distance} = 0.1\ \text{m}$$

$$FDA = \text{Frequency Dependent Attenuation (dB m}^{-1}\text{)}$$

Click detection distances were calculated for 14 *Y. evonymella*, nine *Y.cagnagella*, and one *Y. padella*. Directional click detection distance was calculated in the same manner but using ten consecutive wingbeats per angle (0°, 45°, 90°, and 180° for *Y. evonymella* (n=8), with the addition of 135° for *Y. cagnagella* (n=8)).

2.3.7 Echo measurements

I used a technique known as acoustic tomography to measure the echo strengths of five *Yponomeuta evonymella* in the form of their spectral target strength (the amount of the original signal returned from the target). A sonar measurement head, replicating the mouth and ears of a bat head, consisting of a ¼" ultrasound microphone (type 40BE), pre-amplifier (26AB, both GRAS Sound & Vibration A/S, Holte, Denmark), dual-channel microphone power supply (type 5935-L, Brüel & Kjær, Nærum, Denmark) and a custom-made ferro-electret foil loudspeaker (33x14mm, Emfit Ltd., Vaajakoski, Finland) powered by a PZD350 M/S high-voltage amplifier (TREK Inc., Lockport, NY), was mounted on a lever arm, which was rotated vertically by a LT360 turntable (LinearX Systems Inc., Battle Ground, WA). For frequency responses of the microphone and speaker see Appendices 4 and 2 respectively. The target insect was placed on a vertical tower (27.8 cm high) of ultrasound absorbing foam (Basotect W, BASF, Ludwigshafen, Germany) on a horizontally rotating turntable (LT360, LinearX Systems Inc., Tualatin, OS), and the sonar measurement head was positioned ~31 cm (this varied slightly but was controlled for between measurement sessions) from the target with both the microphone and speaker separated by 15 mm and facing the target insect. The turntable, speaker, and microphone were connected to a NI-DAQ BNC-2110 soundcard controlled using custom-programmes through LabVIEW v.16.0 (both National Instruments, Austin, TX). A frequency modulated sweep from 15 to 250 kHz was used to ensonify the insect and for each position four echoes were recorded and averaged. Refer to Figure 2.4 for a not-to-scale schematic of the set-up.

Acoustic tomography takes echo 'slices' of a target by measuring the incident echo intensity at different angular increments and then software (custom MATLAB script) is used to piece these slices together. This process allows an image of a target to be created purely based on its echoic properties, a sound image. It is then possible to isolate regions of the target and extract their echo strengths for analysis, echoic properties can then be calculated, for instance spectral target strength or detection distance (by a bat). By mimicking a bat head (ears and mouth) with a microphone and ultrasonic loudspeaker respectively, the data gathered by this technique represent the sensory input of bat, thus; subsequent perceptual space modelling of a target is accurate for bat-insect interactions.

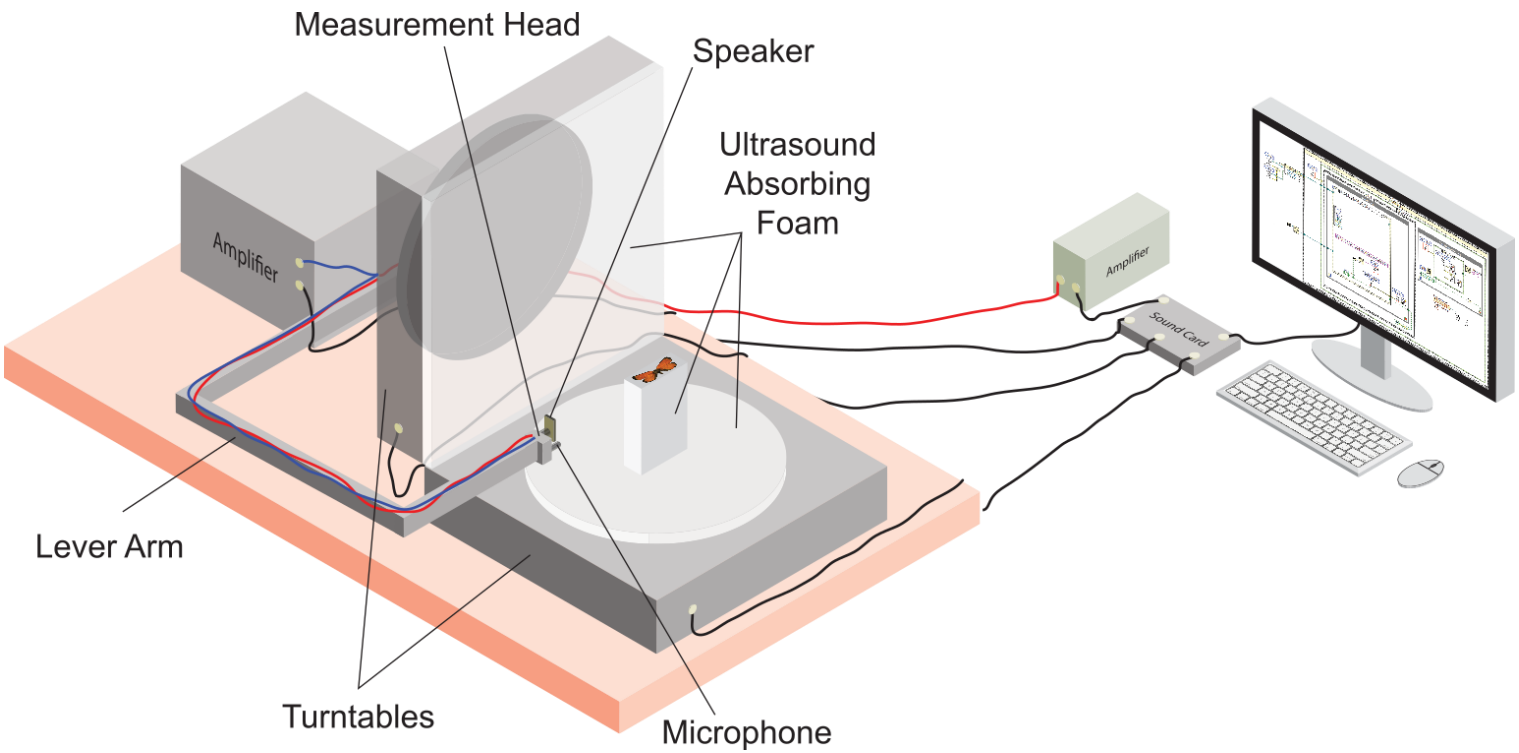


Figure 2.4 Acoustic tomography schematic. Not-to-scale schematic of the acoustic tomography set-up used in Chapter 2 & 4. Black lines represent cables connected to the sound card, the red line represents the cable connecting the microphone to the Brüel & Kjær microphone power supply and amplifier (marked "Amplifier" and next to the "Sound Card"), and the blue line represents the cable connecting the speaker and its "Amplifier" (behind the turntable). The white surfaces represent ultrasound absorbing foam on the turntables. The vertical turntable is 20% transparent in order to reveal the rotating part on its reverse, which was attached to, and rotated, the lever arm and measurement head over the specimen. The measurement head consisted of an ultrasonic microphone and speaker. The whole set-up was controlled through a custom LabVIEW script.

Five *Y. evonymella* were analysed using acoustic tomography. Each individual was set with their wings held vertically with the leading edge of the forewing perpendicular to the longitudinal axis of the body. The moth was measured from 0-180° in 0.5° steps in the horizontal plane at an elevation angle of 0°. A frequency modulated sweep from 15 to 250 kHz was used to ensonify the moth and for each position four echoes were recorded and averaged.

Detection distance of echoes was calculated analogous to click distance (see above, but for two-way spherical transmission losses and with FDA for bat call frequencies with the highest detection range in the UK, i.e. at 20-30 kHz:

$$BSL - 2 * 20 * log_{10}\left(\frac{\delta - \delta ref}{\delta ref}\right) - 2 * FDA * (\delta - \delta ref) + TS = HT$$

$TS =$ spectral target strength of moth echo (dB at δref)

$BSL =$ Source Level of bat call (dB peSPL at δref)

2.3.8 Statistics

All statistical tests were performed using R studio (R version 3.1.2.). A two-tailed paired samples t-test was performed to compare the number of clicks produced before and after ablation of the aeroelastic tymbals. A two-tailed nested ANOVA run as a mixed effects model, with moth individual as the random effect, was used to test for differences between the amplitudes of *Yponomeuta evonymella* and *Y. cagnagella* sounds recorded at different angles. Moth individual was nested within the angle at which it was recorded. This was followed by a pairwise Tukey post-hoc test with Bonferroni correction for each species.

2.4 Results

2.4.1 *Yponomeuta* produce ultrasonic clicks in flight using their hyaline patches

I recorded *Yponomeuta evonymella*, *Y. cagnagella* and *Y. padella* in free (*Y. evonymella* only) and tethered flight. All 27 tested individuals (15 tethered and two free flying *Y. evonymella*, nine tethered *Y. cagnagella*, and one tethered *Y. padella*) produced two bursts of a similar number of broadband ultrasonic clicks for every wingbeat cycle (Figure 2.5 Figure 2.6). One burst was produced at the beginning of the upstroke (lower burst) and the other at its end (upper burst), with the clicks emitted in a more rapid succession during the former. The number of clicks per burst appears to mirror the number of striations on the hyaline patch. In *Y. evonymella* the mean number of clicks per burst was 12.6 (± 1.7, n=14) and the number of striations was 11+ (Figure 2.2b). Note however that these recordings are a superposition of the click bursts created by the two hindwings, as proven by almost identical sounds recorded with microphones on either side of the moth.

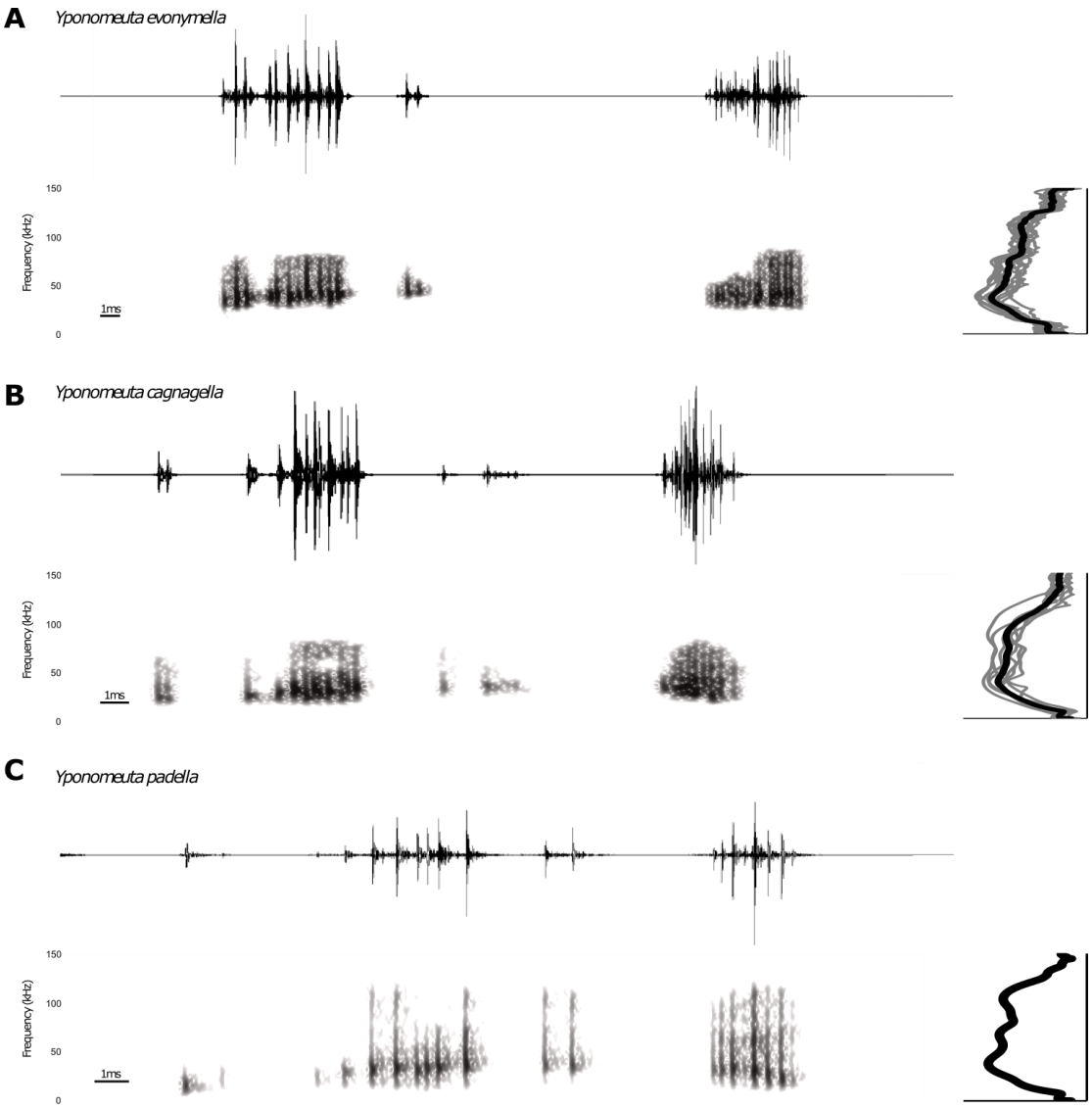


Figure 2.5 Spectral and temporal characteristics of *Yponomeuta* sounds The waveform and spectrogram (FFT size 1024, window FlatTop, overlap 25%) of typical examples of in-flight acoustic emissions of three species of *Yponomeuta*, (A) *Y. padella*, (B) *Y. evonymella*, and (C) *Y. cagnagella*. Each plot represents one full wingbeat showing the two bursts of clicks produced with each wingbeat cycle. To the right of each spectrogram is a power

spectrum showing the normalised click amplitude for the species mean (black line) and individuals (grey lines, for each species n see methods). Time scales vary between plots, and spectrograms are not calibrated for amplitude.

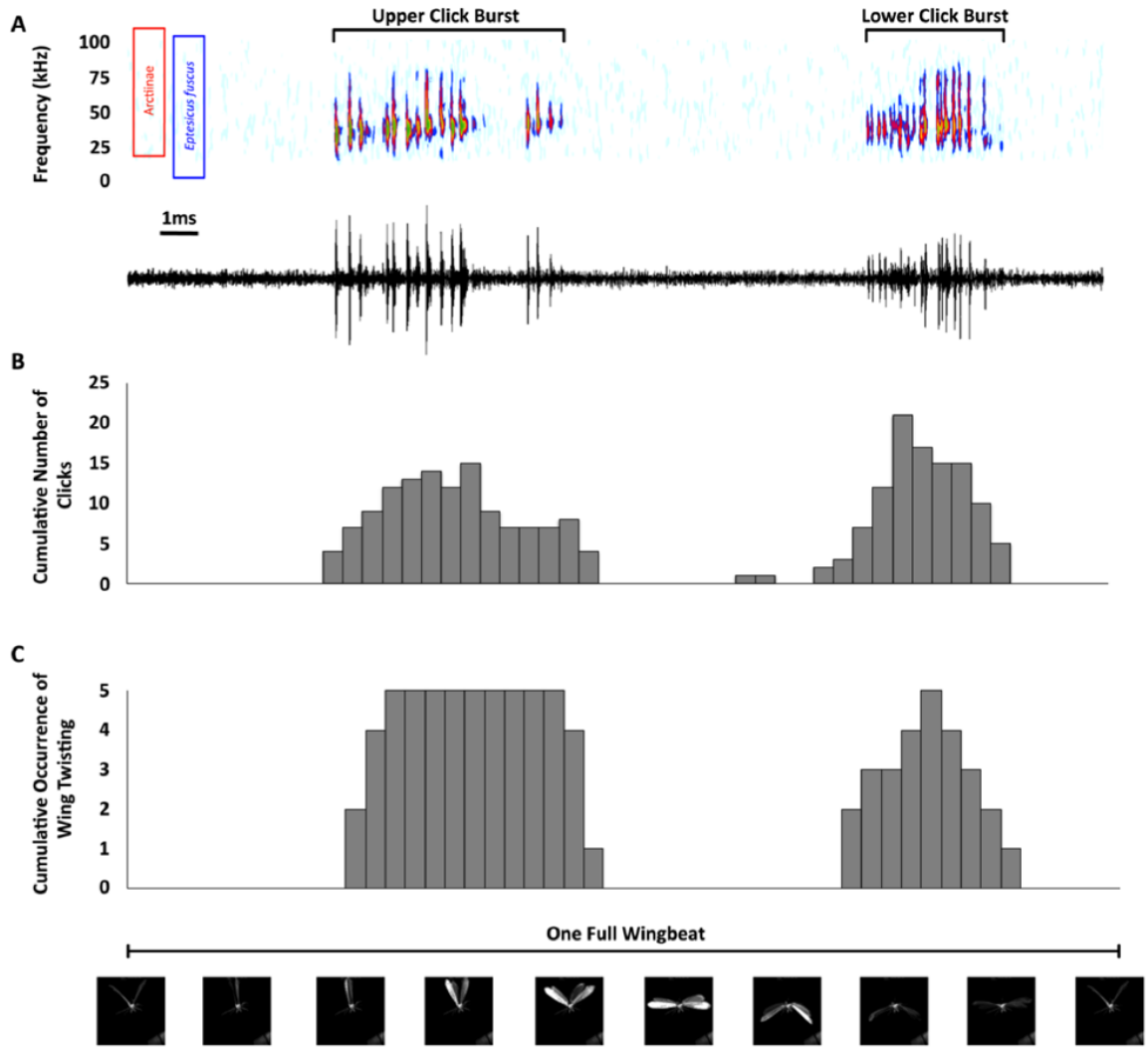


Figure 2.6 Synchronisation of click bursts with wing beats in *Yponomeuta evonymella*. (A) Spectrogram (FFT size 256, window Hamming, overlap 25%) and waveform showing an example of the two bursts of ultrasonic clicks produced during one wingbeat. Red and blue boxes represent the frequency range of arctiine anti-bat sounds (Corcoran et al., 2010) and the frequency hearing range of *Eptesicus fuscus* (an insectivorous bat) (Koay et al., 1997) respectively. (B) Histogram of the number of clicks produced over five consecutive wingbeats from one individual, fifty time bins were made for each wingbeat, each bar represents the total number of clicks for the corresponding bin from all five wingbeats. (C) Histogram of the occurrence of twisting at the wing joint over the same five consecutive wing beats as panel B, a value of 1 was assigned for the occurrence and 0 for absence of twisting, 50 time bins were made each lasting two frames, each bar represents the total occurrence of twisting for the corresponding bin from all five wingbeats. Stills from a high-speed video (3000 fps) of *Y. evonymella* show how plots A-C relate to the moth's wingbeat. All plots represent one full wingbeat; however, both plots in panel A represent the same individual wingbeat and have the same time scale.

2.4.2 Characterisation of the acoustic emissions of three species of *Yponomeuta*

Ten clicks recorded laterally (90°) from each of 14 *Y. evonymella*, nine *Y. cagnagella* and one *Y. padella* were analysed for duration, temporal, amplitude and spectral parameters (Table 2.1). To measure horizontal emission directionality eight *Y. evonymella* (only six for 45°) were recorded from 0°, 45°, 90°, and 180° and five clicks analysed each (n=150). Additionally, eight *Y. cagnagella* were analysed from 0°, 45°, 90°, 135° and 180° and 10 clicks analysed each (n=400).

Table 2.1 Acoustic properties of three *Yponomeuta* species Acoustic properties (mean ± SD; n= clicks) of the clicks of *Yponomeuta evonymella* (14 individuals), *Y. cagnagella* (nine individuals) and *Y. padella* (one individual). For each of 10 consecutive wingbeat cycles the highest amplitude click was selected. From its waveform the source level, and from the spectrum (Hamming window size 1024) peak, low and high (highest and lowest frequency 15 dB below the amplitude of the peak frequency) frequencies were measured. Click detection distance was calculated from source levels and peak frequencies using an adaptation of the sonar equation, including frequency dependent attenuation (see methods). Ten wingbeat cycles (20 bursts) were analysed for click duration, duty cycle and the number of clicks per burst (see methods).

Species	Source Level (dB peSPL 0.1 m)	Peak Frequency (kHz)	Low Frequency (kHz)	High Frequency (kHz)	Click Detection Distance (m)	Click Duration (µs)	Lower Burst Click Duration (µs)	Upper Burst Click Duration (µs)	Duty Cycle (%)	Number of Clicks per Half Modulation Cycle (Burst)
Yponomeuta evonymella	57.5 ± 2.6 (n=140)	37.8 ± 5.6 (n=140)	23.1 ± 2.5 (n=140)	67.9 ± 9.7 (n=140)	8.1 ± 0.6 (n=140)	26.3 ±3.3 (n=280)	27.4 ± 2.5 (n=140)	25.2 ± 3.3 (n=140)	1.9 ± 0.4 (n=280)	11.9 ± 1.1 (n=280)
Yponomeuta cagnagella	64.5 ± 0.6 (n=90)	43.5 ± 3.9 (n=90)	21.2 ± 1.8 (n=90)	97.1 ± 2.9 (n=90)	10.5 ± 0.8 (n=90)	28.9 ±3.6 (n=180)	26.6 ± 6.7 (n=90)	31.1 ± 5.0 (n=90)	3.4 ± 0.8 (n=180)	18.8 ± 4.1 (n=180)
Yponomeuta padella	58.2 ± 0.3 (n=10)	37.2 ± 0.5 (n=10)	18.0 ± 0.7 (n=10)	117.1 ± 0.9 (n=10)	8.3 ± 0.1 (n=10)	32.8 ±1.0 (n=10)	34.0 ±1.9 (n=20)	33.4 ± 1.7 (n=20)	3.1 (n=10)	12 (n=20)

2.4.3 Ablation

I removed both tymbals (area 260x800 µm; see Figure 2.2) in 12 tethered *Y. evonymella*, recorded their flight sounds pre- and post-ablation, and determined the number of clicks produced per 100 ms (about three wingbeat cycles) as this is the duration used in the literature for calculating parameters such as maximum duty cycle (Corcoran et al., 2010). Post-ablation, seven individuals produced no clicks, the eighth individual produced one click, and the remaining four produced fewer clicks with lower amplitudes. Microscopic examination showed that ablation of the hyaline patch had been incomplete in these four individuals, so these were excluded from further analysis. A paired-samples t-test revealed a highly significant difference (n=8, $t(7)=20.3$, $p<0.001$) between the two treatments (Figure 2.7).

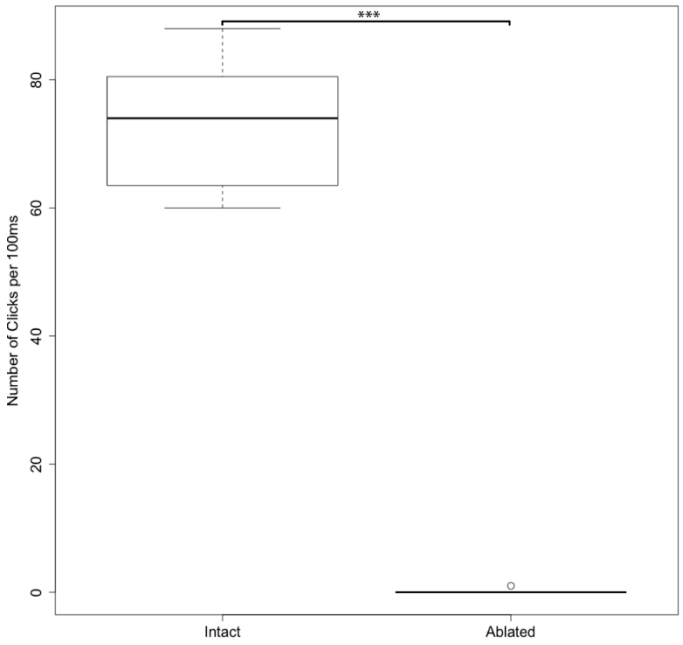


Figure 2.7 Ablation tests The number of clicks produced over a 100ms period of flight of eight *Yponomeuta evonymella* individuals pre- and post-ablation. Asterisks (three indicating p<0.001) indicate a significant result of a paired-samples t-test between the two treatments. Whiskers represent 1.5*IQR, and open circles represent outliers.

2.4.4 Hearing tests

Twenty *Y. evonymella* and four *Y. cagnagella* were used in hearing experiments. While in flight, no individual of either species reacted to the playback of an ultrasonic pulse known to elicit reactions of moths possessing ultrasonic hearing (Juliana et al., 2007). There was no flight cessation, or even alteration in flight direction. The 20 *Y.*

evonymella individuals were exposed to the stimulus while resting as a group within a flight cage, as were the four *Y. cagnagella*, in a separate cage. None of these resting individuals showed any response, such as twitching, movement cessation, or flight initiation to ultrasound playback. Ten *Y. evonymella* were also left in a flight cage and their responses to each other observed. As with the playbacks, no individuals showed any change in resting behaviour in response to take-off or flight, and therefore sound production, of any other moth.

2.4.5 Proposed mechanism of sound production

High-speed infrared videos of *Y. evonymella* and *Y. cagnagella* in tethered flight revealed that there was no contact of any body part (potentially serving as scraper) with the hyaline patch during sound production or any other phase of the wingbeat cycle. So *Yponomeuta* do not produce sound by stridulation. Instead, clicks exclusively and always occur while the hindwing rotates (pronates or supinates) along its base-to-tip axis during the upper and lower turning phases of a wing stroke (Figure 2.6 and see Supplementary Video S1 from O'Reilly et al. (2019)). More detailed analysis shows that during supination at the beginning of the upstroke the posterior anal and jugal areas of the hindwing fold downwards relative to its anterior remigium, along what is likely the claval furrow (Figure 2.8).

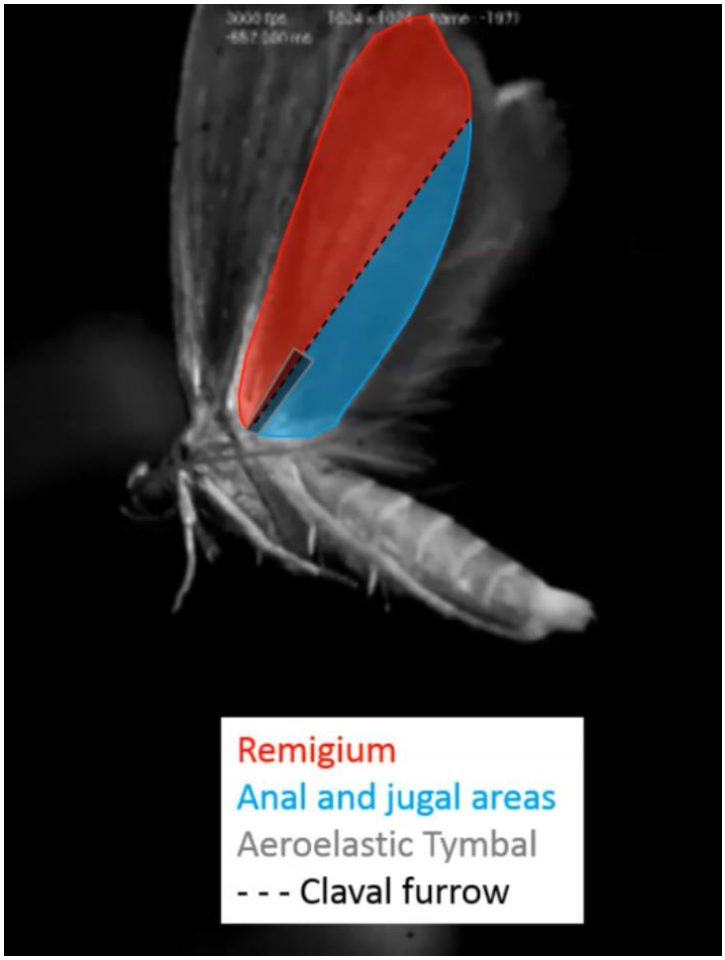


Figure 2.8 *Yponomeuta cagnagella* wing morphology Regions of the hindwing labelled on *Yponomeuta cagnagella*.

This folding progresses from the tip to the base of the wing including the hyaline patch, and its folding coincides with the production of the lower click burst (see supplementary video 2 O'Reilly et al., (2019)). During pronation at the top of the upstroke the upper click burst is produced, but no equally obvious folding of the hindwing occurs (Figure 2.6).

2.4.6 Detectability of *Yponomeuta* by echolocating bats

In terms of the horizontal directionality of *Y. evonymella* clicks, pairwise comparisons following a nested ANOVA (n= 264, $F(1,3)= 7145.475$, $p<0.001$) showed that the sounds recorded laterally were significantly louder than those recorded at the three other angles (0° and 90°, $Z=6.6$, $p<0.001$, 45° and 90°, $Z=5.8$, $p<0.001$, and 180° and 90°, $Z=-9.0$, $p<0.001$) (Figure 2.9). There were no differences between the other three orientations (0° and 45°, $Z=0.3$, $p=1.0$, 0° and 180°, $Z=-2.3$, $p=0.13$, 45° and 180°, $Z=-2.4$, $p=0.11$) (Figure 2.9). Mean estimated distances over which bats can detect these

clicks were 6.0 ±0.4 m (n=8, 40) at 0°, 6.5 ±0.4 m (n=6, 30) at 45°, 7.9 ±0.7 m at 90°, and 5.6 ±0.4 m at 180° (n=8, 40) (Figure 2.11b).

As with *Y. evonymella*, *Y. cagnagella* amplitudes were compared by orientation using a nested ANOVA (n=640, $F(1,4)=6321.408$, $p<0.001$). The following pairwise comparisons showed that sounds recorded from all angles were different barring 180° and 45°. The results are as follows (Figure 2.10): 0° and 45°, $Z=5.5$, $p<0.001$, 0° and 90°, $Z=17.4$, $p<0.001$, 0° and 135°, $Z=11.0$, $p<0.001$, 0° and 180°, $Z=7.3$, $p<0.001$, 45° and 90°, $Z=11.9$, $p<0.001$, 45° and 135°, $Z=5.5$, $p<0.001$, 45° and 180°, $Z=1.8$, $p=0.68$, 90° and 135°, $Z=-6.4$, $p<0.001$, 90° and 180°, $Z=-10.0$, $p<0.001$, 135° and 180°, $Z=-3.7$, $p=0.002$.

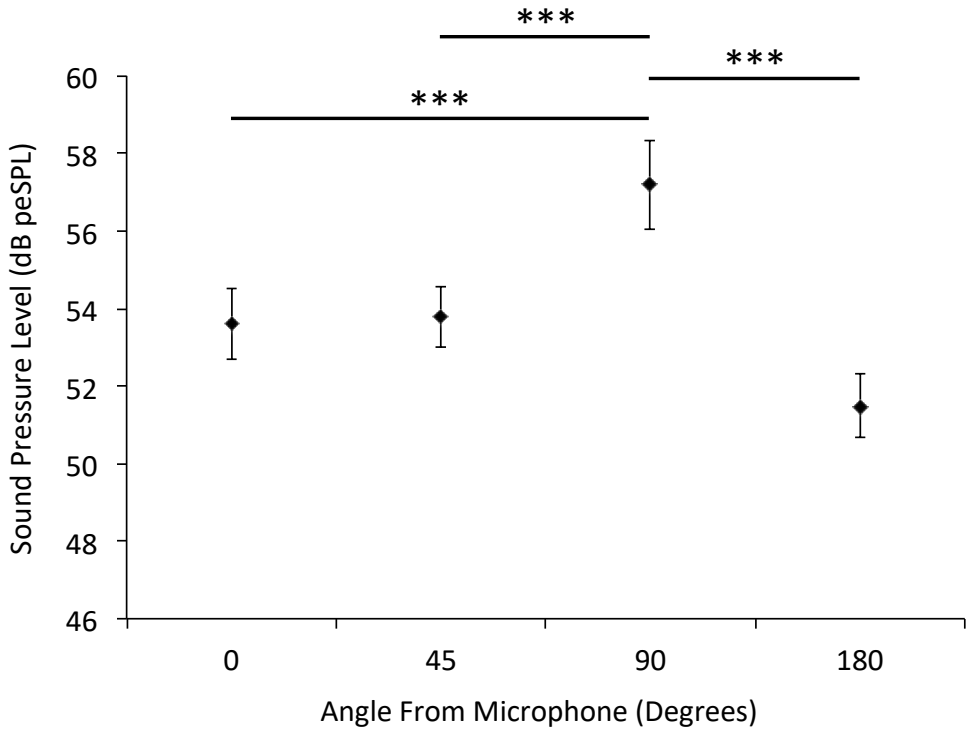


Figure 2.9 Sound pressure level directionality of *Yponomeuta evonymella* clicks Mean source level (in dB peSPL re 0.1 m) of eight *Y. evonymella*, recorded from four different directions, 0° corresponds to the microphone positioned anteriorly to the moth, 180° posteriorly, 90° laterally, and 45° anterio-laterally. Asterisks indicate significant differences of nested ANOVA using a mixed effect model with a Bonferroni-corrected Tukey post-hoc test. Error bars show standard deviation.

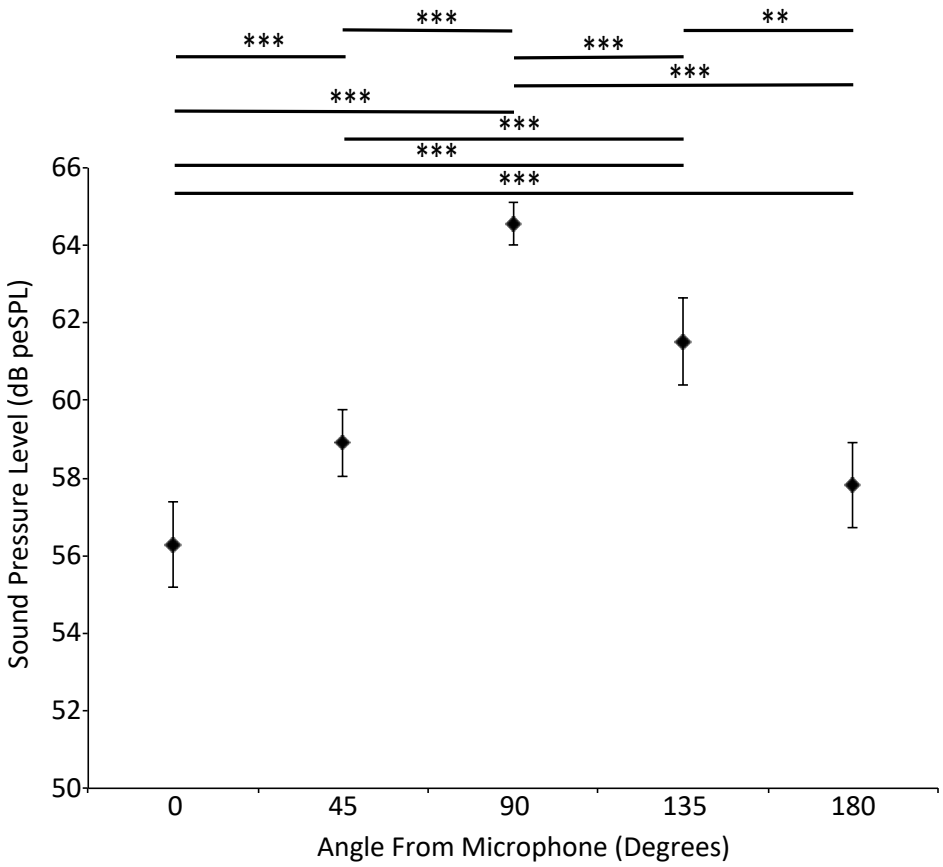


Figure 2.10 Sound pressure level directionality of *Yponomeuta cagnagella* clicks Mean source level (in dB peSPL re 0.1 m) of eight *Yponomeuta cagnagella*, recorded from five different directions, 0° corresponds to the microphone positioned anteriorly to the moth, 180° posteriorly, 90° laterally, 45° anterio-laterally, and 135° posterior-laterally. Asterisks indicate significant differences of nested ANOVA using a mixed effect model with a Bonferroni-corrected Tukey post-hoc test. Error bars show standard deviation.

Echo spectral target strengths of five *Y. evonymella* were measured to determine over what distances they would be detectable to the biosonar of insectivorous bats. Spectral target strength (the sound pressure reflected back to the receiver compared to the incident amplitude at each frequency) ranged between -35 and -43dB at all frequencies between 20 and 160 kHz (Figure 2.11a). Total target strength was highest when the moth was at 90° to the bat corresponding to a mean detection distance of 7.1 ±1.1 m (n=5) for frequencies between 20-30 kHz (the most ecologically relevant frequencies for British bats), and at its lowest when it was 177° to the bat with a mean detection distance of 4.3 ±0.8 m (n=5) at these frequencies (Figure 2.11b).

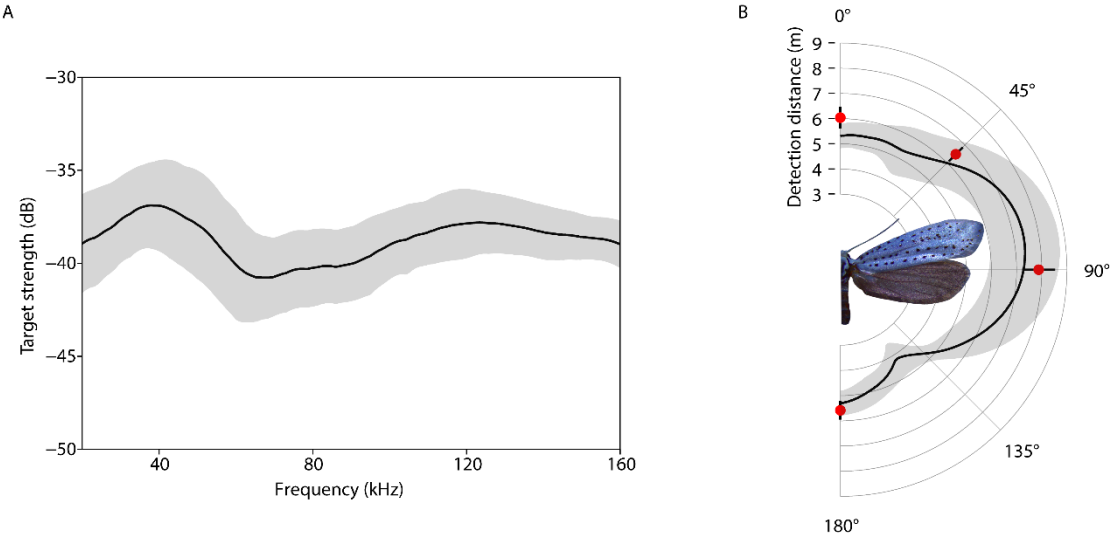


Figure 2.11 Echo strength and detection ranges (A) Spectral target strength (black line: mean, grey area: SD, n=5) of five dried *Y. evonymella* with the wings in an upright position to represent a mid-flight moth, as this will produce the loudest (most ecologically relevant) echoes a bat would receive. (B) Directionality of detection distance of the right-hand side of *Yponomeuta evonymella* based on echo target strengths over 180° in 0.5° steps (black line: mean, grey area: SD, n=5), and sound produced by the moth's wing-based tymbal during tethered flight in four orientations; 0°, 45°, 90°, and 180° (red circles: mean, error bars SD, n=8, 80). Photo of *Yponomeuta evonymella* copyright L. J. O'Reilly 2018.

2.4.7 Comparisons of *Yponomeuta* and Arctiinae sounds

Published Arctiinae (Erebidae) anti-bat sounds were used as a comparison to aid in inferring a possible function of *Yponomeuta* sounds. These moths were chosen due to the extensive research into their acoustics, as well as the structural similarities of the *Yponomeuta* hyaline patches and arctiine tymbals.

Temporal pattern, spectral information, and duty cycle were compared with arctiine sounds from the literature (Conner and Corcoran, 2012; Corcoran et al., 2010). Both *Yponomeuta* sounds and arctiine anti-bat sounds are broadband ultrasonic clicks (Corcoran et al., 2010). *Yponomeuta* sounds fall within the frequency range of known arctiine anti-bat sounds, as well as the hearing range of echolocating bats (Figure 2.6a). However, *Yponomeuta* sounds have lower mean amplitudes (*Y. evonymella* 57.5 ±2.6dB peSPL, *Y. cagnagella* 64.5 ±0.6dB peSPL, *Y. padella* 58.2 ±0.3dB peSPL) than those of the Arctiinae (80.3dB peSPL from Corcoran et al. (2010)). Additionally, the trains of *Yponomeuta* clicks increase and decrease in peak frequency over time similarly to the Arctiinae (Figure 2.12). Overall, *Yponomeuta* sounds show many similarities to arctiine anti-bat sounds.

Corcoran *et al.* (2010) showed that duty cycle and the number of clicks per modulation cycle can be used to group arctiine anti-bat sounds by how they function, either by jamming bat biosonar or providing an aposematic signal. If *Y. evonymella*, *Y.*

cagnagella and *Y. padella* are similarly plotted, they do not fall into either group; their duty cycles are too low to be included as sonar jammers, and the number of clicks they produce per modulation cycle (per two buckling events, i.e. per full wingbeat) is too high to be included as an aposematic signaller or mimic based on Corcoran *et al*. (2010) (Figure 2.13).

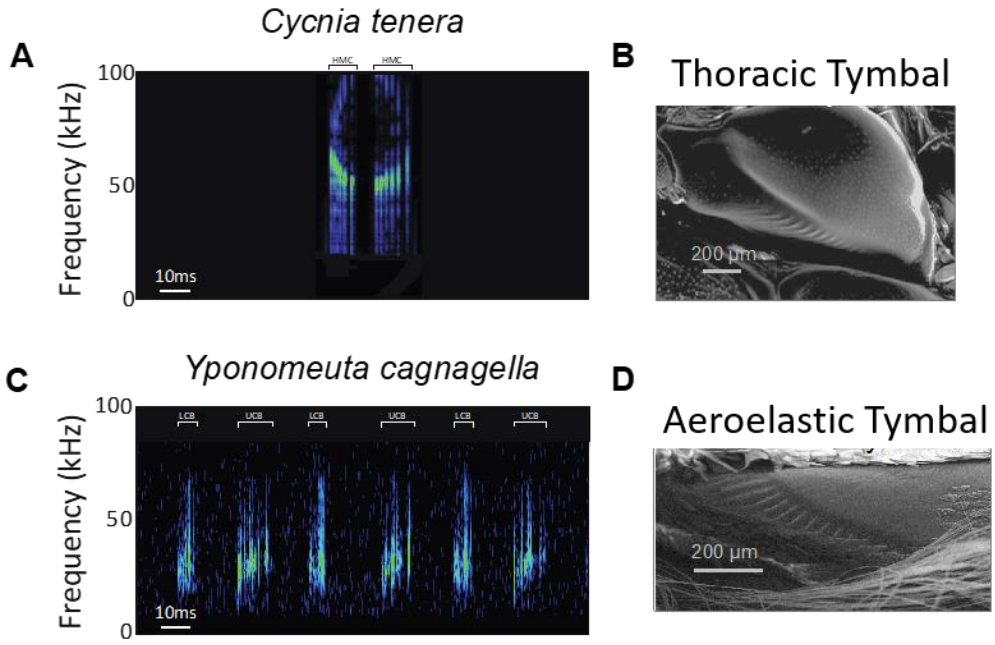


Figure 2.12 Acoustic and structural convergence of *Yponomeuta* and Arctiinae sound production (A) Spectrogram of *Cycnia tenera* (Arctiinae) demonstrating the decrease and then increase in pitch of the two half modulation cycles (HMC) or click bursts (B) SEM of the thoracic tymbal of *C. tenera*. (C) Spectrogram (FFT 256, window Hamming, overlap 25%, not calibrated for amplitude) of sounds produced during three full wingbeats of *Yponomeuta cagnagella* showing the decreasing pitch of the click burst produced during the lower phase (Lower Click Burst, LCB) of the wingbeat and the subsequent increasing pitch of the click burst produced during the upper phase (Upper Click Burst, UCB). (D) SEM of aeroelastic tymbal of *Y. cagnagella*.

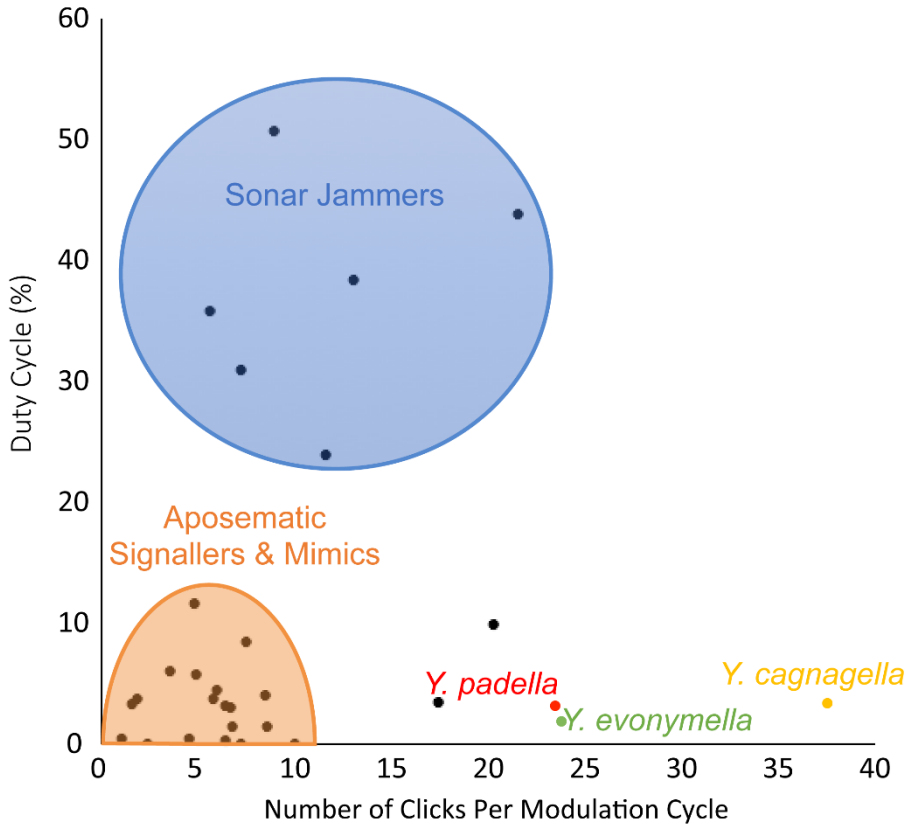


Figure 2.13 Comparison of the potential function of *Yponomeuta* sounds to the anti-bat sounds of the Arctiinae
Maximum duty cycle (the percentage of time an acoustic signal is on) against the number of clicks per modulation cycle (the number of clicks per bucking in and out of a tymbal) is a plot which can be used to group Arctiinae species by the anti-bat function of their sounds, either aposematism (or associated mimicry) or jamming (Corcoran et al. 2010). 27 arctiine species (black markers) are plotted from Corcoran *et al.* (2010) along with three *Yponomeuta* species (coloured markers), *Y. cagnagella* (n=14), *Y. evonymella* (n=9), and *Y. padella* (n=1).

2.5 Discussion

2.5.1 The hyaline hindwing patches of *Yponomeuta* likely act as buckling tymbals

Several lines of evidence corroborate the hyaline patch as a buckling tymbal. First, hyaline patch structure is strikingly convergent to the ultrasound emitting tymbals found on the thorax of many arctiine moths (Blest et al., 1963; Fenton and Roeder, 1974) (Figure 2.12). In both, similarly sized thin areas of cuticle, with air on either side, consist of a larger smooth area (window) with a series of parallel striations of increasing length (band of microtymbals) running alongside it (Figure 2.2). In arctiines, an inward muscular pull buckles the microtymbals in sequence and creates a burst of individual clicks. When the muscle relaxes, elastic forces buckle the microtymbals back in the reverse order creating a similar second burst of clicks (Blest et al., 1963; Fullard and Heller, 1990). Secondly, the peak frequency of individual clicks increases during one

burst and then decreases in the other, which is in agreement with the reverse order of buckling in and out (see Figure 2.12 and Barber *et al.* (2009) and Corcoran *et al.* (2010)). Almost identically, *Yponomeuta* sounds also consist of two alternating click bursts with concurrent increases and decreases in individual click peak frequencies (Figure 2.6a and Figure 2.12). Additionally, the mean number of clicks per burst (Table 2.1) is just above the number of striations (Figure 2.2). Note though that the tymbals in the two hindwings operate in parallel, thereby theoretically creating twice as many clicks per burst as there are striations. My recordings show that the moth body does not cast an effective sound shadow; the clicks from both hindwings reach to either side. I propose that the observed mean number of clicks per burst is only about half the theoretical maximum for both wings combined because many clicks will coincide between sides, some neighbouring striations might buckle together, and some clicks may be too faint to be detected amongst other louder clicks. Interestingly, should clicks from both sides occur simultaneously, this may result in constructive interference of the sound waves, thus doubling the amplitude of the clicks. Vice versa, should the tymbals be activated asynchronously, this may result in a greater number of lower amplitude clicks as they are not benefiting from constructive interference. In summary, I conclude that the striations act as microtymbals, and that they are convergent in structure and mechanism to the sound production by microtymbals of arctiines. It can even be speculated that the tymbal deformations leading to microtymbal buckling might be similar.

The actuation mechanisms creating these tymbal deformations are however fundamentally different. In arctiines, direct muscle actuation deforms the tymbal, while in *Yponomeuta*, flight muscles at the base of the wing are the actuators, and the tymbal is deformed by the rotation and aeroelastic folding of the hindwing along the claval furrow (a natural folding directly adjacent to the microtymbals of the hyaline patch) during the wingbeat cycle (see Supplementary Videos S1 and S2 from O'Reilly et al. (2019)). Because the actuation of the hyaline patch is due to aerodynamic forces and the aeroelastic properties of the wing, I am terming it the 'aeroelastic tymbal'. The evidence supports that the claval furrow is integral to the actuation of tymbal buckling, the exact biomechanical buckling mechanism is still unclear though and is beyond the scope of this project as it requires complex biomechanical modelling to fully elucidate it.

2.5.2 *Yponomeuta* acoustically mimic arctiine anti-bat warning sounds

In addition to the structural convergence of the aeroelasctic tymbal of *Yponomeuta* and thoracic arctiine tymbals, the sounds they produce are also similar. All three species of *Yponomeuta* produced two bursts of ultrasonic clicks similar to those of the Arctiinae, with peak frequencies within both the hearing range of bats and the range of frequencies produced by arctiines, including sympatric species *Arctia caja* and *Phragmatobia fuliginosa* (16.6 to 109.5 kHz; Figure 2.6a)(Corcoran et al., 2010; Koay et al., 1997; Surlykke and Miller, 1985). In conjunction with the lack of hearing, and therefore lack of acoustic intraspecific communication, this suggests an anti-bat function. However, the remarkable acoustic difference to arctiines is that *Yponomeuta* sounds are produced constantly during flight. All other Lepidoptera produce sound only at specific times, for example during courtship, territory defence, or in response to the perceived presence of bats (Bailey, 1978; Conner and Corcoran, 2012; Nakano et al., 2009). Perpetually casting its protective sound signal is advantageous for a deaf moth unable to detect and react to approaching bats.

Yponomeuta sounds appear to be a defence directed at bats, but to what effect? The constant nature of *Yponomeuta* sound production renders it unlikely that startle is the main mechanism of defence, as bats may habituate to these sounds and even use them as cues to find prey (Bates and Fenton, 1990). Arctiine anti-bat sounds used for aposematism/mimicry differ characteristically from those for sonar jamming in their maximum duty cycle (the percentage of time a signal is 'on') and the number of clicks per modulation cycle (Corcoran et al., 2010). Whilst *Y. evonymella, Y. cagnagella* and *Y. padella* produce more clicks per modulation cycle than typical aposematic signalling arctiines, their duty cycles of 1.9, 3.4 and 3.1% respectively place them exactly within the range of aposematic signalling arctiines (Figure 2.13). These low duty cycle anti-bat sounds are unable to jam biosonar, as a duty cycle of 20% or more is essential (Conner and Corcoran, 2012; Corcoran et al., 2010). Whilst plotting sounds using these parameters is useful in suggesting their function, as the analysis was run using only the Arctiinae data, the absence of *Yponomeuta* from either of the groups does not mean they do not function in one of these ways. I believe that the risk of a distasteful or potentially harmful meal would mean that bats likely generalise low duty cycle broadband click trains as indications of unpalatability. I hence conclude that *Yponomeuta* are acoustically mimicking the aposematic Arctiinae.

2.5.3 *Yponomeuta* employ acoustic Müllerian mimicry

The toxicity or unpalatability of an organism indicates whether their mimicking warning signals are truly aposematic (Müllerian mimicry) or impostors (Batesian mimicry) (Bates, 1862; Müller, 1879). *Yponomeuta* larvae tend to be monophagous or at least limited to only a few species of food plant (Menken et al., 1992), something often associated with Lepidoptera that sequester specific toxins (Engler-Chaouat and Gilbert, 2007). Principally, their hosts tend to be from Celastraceae, Rosaceae, Salicaceae and Crassulaceae (Menken et al., 1992). Celastraceae and Crassulaceae contain butenolides (Fung et al., 1988), secondary metabolites the derivatives of which are reported to have cytotoxic activity (Wagner et al., 1981), and Salicaceae contain salicin, a secondary metabolite known to act as a deterrent to insects and mammals (Bernays et al., 2000; Menken et al., 1992; Pass and Foley, 2000). *Yponomeuta cagnagella* larvae feed on *Euonymus europaeus* (European spindle tree; Celastraceae) which contains two butenolides, siphonodin and to a lesser extent isosiphonodin (Fung et al., 1988). Isosiphonodin is found in *Y. cagnagella* and is either synthesised or sequestered by the insect (Fung et al., 1988). Interestingly, isosiphonodin is also found in adults of *Yponomeuta* species that do not feed on butenolide-containing plants (Fung et al., 1988), providing evidence that at least these moths synthesise butenolides. Synthesis of isosiphonodin in several species of *Yponomeuta* suggests that the compound is important in the ecology of these insects. Additionally, *Prunus padus* (Bird cherry, Rosaceae), the food plant of *Y. evonymella*, contains glucosides that can release hydrogen cyanide upon digestion, which has led to cattle poisoning (Sargison et al., 1996).

Unpalatability to predators is an obvious proposal for a function of containing butenolides and other noxious compounds. In fact, birds became drowsy when force-fed *Yponomeuta* adults (Menken et al., 1992). However, Menken *et al.* (1992) described neither larvae nor adults of *Yponomeuta* as obviously visually aposematic. Instead I show that their aposematic signals are targeted towards bats and are acoustic, not visual. These moths produce sounds with properties extremely similar to the aposematic signals of larger moths (particularly arctiines), and are mostly nocturnal and therefore at low risk of predation by birds, explaining the lack of visual aposematism, as is the case in aposematic Arctiinae (Ratcliffe and Nydam, 2008). So, these butenolides, and probably other compounds such as glucosides and salicin, are likely a defence against

bats. I believe that *Yponomeuta* sounds are warning bats of the presence of distasteful and potentially toxic compounds in these moths. Thus, at least the species of *Yponomeuta* containing such compounds are true aposematic signallers and therefore Müllerian not Batesian mimics of arctiines.

2.5.4 *Yponomeuta* sounds do not increase their conspicuousness to hunting bats

The continuous nature of *Yponomeuta* sound production might render them more vulnerable to bats because bats will be able to eavesdrop on and be attracted to the continuously emitted warning sounds. So reduced click amplitude might be adaptive, and *Y. evonymella* clicks are indeed on average around 22dB fainter than those of arctiines (Corcoran et al., 2010). On the other hand, clicks that are too low amplitude might be detected by bats too late to prevent capture. Therefore, the most adaptive warning click would be perceivable over the exact distance that a bat would detect the insect's echoes anyway, and this is what I found for all orientations tested (mean differences of 0.7, 0.5, 0.6 and 0.3 m for 0°, 45°, 90°, and 180° respectively; see Figure 2.11b). Although both click recordings and acoustic tomography were recorded in the lab and not natural conditions, the fact all data were gathered under the same acoustic conditions means that differences are relative to each other. Therefore, although in a natural, noisier environment the absolute values may be lower, I believe the differences, or lack thereof seen here should hold. *Yponomeuta*'s zone of acoustic protection has evolved to be just large enough to cover its zone of detectability by echolocation.

Further supporting the idea that *Yponomeuta* signal amplitudes are an adaptation to their echo detectability, the angular differences in click detection distances are matched in echo detection distance (Figure 2.11b). *Yponomeuta evonymella* click amplitudes are significantly higher laterally when compared to all other orientations, and the fact that this is matched in the echoes is compelling evidence that this has evolved due to selection pressure from bats. For both clicks and echoes, detection distance is highest laterally and lowest anteriorly and posteriorly with anteriolateral orientations being intermediate (Figure 2.11b).

2.5.5 Conclusion

To conclude, *Yponomeuta* and their hyaline patch-possessing relatives produce anti-bat sounds using a completely novel sound-producing structure in the Lepidoptera. Whilst wing-based sound production exists within the order (Agee, 1971; Alcock et al., 1989;

Heller and Achmann, 1993), all other examples are evolutionarily independent of the aeroelastic tymbal and none are used to produce anti-bat ultrasound. *Yponomeuta* use their sounds to mimic the well-studied aposematic anti-bat sounds of the Arctiinae, and it seems likely they are Müllerian mimics. Aeroelastic tymbals are a striking example of both structural and acoustic convergent evolution in the bat-moth evolutionary arms race, as well as being remarkable as a passive acoustic defence mechanism that bypasses the need for predator detection. Clearly, behavioural experiments with bats need to be pursued in order to quantify the protection level these sounds provide. The use of acoustic Müllerian mimicry by a deaf moth in the bat-moth evolutionary arms race shows again how little we know of the complex acoustic war raging in the night skies.

Chapter 3: Multiple convergent evolution of aeroelastic tymbals in the microlepidoptera – phylogeny, anatomy, and acoustics

The Tineidae phylogeny in this chapter was created, under my instruction, by Brogan Harris of the Paleobotany group at the Univserity of Bristol. The *Ethmia bicolorella* acoustic recordings were made in Kenya by Dr David Agassiz of the Natural History Museum, Insect Division under my instruction.

3.1 Abstract

Deaf microlepidoptera of the genus Yponomeuta use a novel wing-embedded aeroelastic tymbal (AT) to produce aposematic anti-bat sound constantly when in flight, negating the need for predator detection, a requirement for other moths which produce anti-bat sounds. Here I assess the phylogenetic spread of ATs within the microlepidoptera, showing that similar structures are widespread in this suborder. By mapping these results to the latest molecular phylogenies, I conclude that ATs have most likely evolved convergently at least 15 times in the microlepidoptera in four different regions of the wings. Incredibly they have evolved independently three times in one subfamily (Tineinae, Tineidae), and I propose the troglophilic (cave-dwelling) and guanophagous ecology of these particular moths, which places them in constant proximity to bats, is responsible for such extreme convergence. I also confirm sound production from, and acoustically characterise, all four AT-possessing taxa I had access to. Their acoustic properties are like those of known anti-bat sounds and as such I suggest that is their function. ATs are an elegant solution to acoustic aposematism for deaf moths and they show that complex acoustic defences are widespread in the understudied microlepidoptera. These small moths are probably an untapped trove of acoustic defences and, thus, require further research with regards to the bat-moth evolutionary arms race.

3.2 Introduction

Since the 1950s, beginning with Roeder's work on anti-bat hearing (Roeder and Treat, 1957), there has been much research interest in the defences of nocturnal moths against bats, and in the bat-moth evolutionary arms race in general. It has been known for decades that many moths have hearing structures to detect bats (e.g. Miller and Surlykke, 2001) and that the Arctiinae produce sounds as a defence against bats functioning through startling their predators, acoustic aposematism, and/or echolocation jamming (e.g. Surlykke and Miller, 1985). However, recently a surge of new discoveries has arisen in this arms race; taxa other than the Arctiinae have been shown to produce anti-bat sounds (Barber and Kawahara, 2013; Corcoran and Hristov, 2014; O'Reilly et al., 2019), the hindwing 'tails' of some moths have been discovered to act as acoustic decoys (Barber et al., 2015; Lee and Moss, 2016), and the acoustic

absorptive power of moth scales has emerged as a fascinating and complex new area of research (Ntelezos et al., 2017; Shen et al., 2018; Zeng et al., 2011). This spate of new discoveries suggests the true extent of moth anti-bat adaptations might substantially exceed current knowledge.

Lepidoptera have been crudely divided by their size into two suborders: the smaller micro- and the larger macrolepidoptera. The vast majority of research into the anti-bat defences of moths has focussed on the macrolepidoptera, yet preferred prey size varies greatly both within (Waters et al., 1995) and between bat species. Some species such as *Myotis septentrionalis*, rely heavily on microlepidoptera as dietary constituents (e.g. Dodd et al., 2012). Microlepidoptera are therefore also under significant predation pressure from bats.

It would seem highly likely that such pressure on the microlepidoptera would also lead to the evolution of anti-bat defences. However, research into such defences has seemingly just recently begun, with only two studies, other than those investigating the well-known pyralid hearing (e.g. Skals and Surlykke, 2000), addressing the subject. Firstly, Kovalev (2016) suggested that the feather-like wing plumes of *Alucita hexadactyla* (Alucitidae) may have evolved to reduce its echo intensity, and secondly O'Reilly et al. (2019) (see Chapter 2) discovered that the hyaline (transparent) hindwing patches of the genus *Yponomeuta* (Yponomeutidae) are wingbeat-powered aeroelastic tymbals (ATs) that render these deaf moths acoustic Müllerian mimics of aposematic Arctiinae.

Yponomeuta ATs produce two bursts of ultrasonic clicks through buckling of striations with every wingbeat, one burst per wing stroke. As these moths are deaf and unable to detect hunting bats and subsequently acoustically warn them, these structures allow them to bypass predator detection and constantly produce warning sounds. *Yponomeuta* provide the first example of constitutive acoustic aposematism in the bat-moth arms race. This is an elegant evolutionary solution for unpalatable, deaf microlepidoptera and is unlikely to be exclusive to the Yponomeutidae. It is very probable that many microlepidopteran species possess yet undocumented defences against bats, and here I specifically investigate the recently discovered ATs.

Given the AT of *Yponomeuta* reveals itself as a hyaline patch in the wing, presence of hyaline patches in other microlepidopteran taxa might suggest similar acoustic

functionality. The presence of hyaline wing patches is indeed not exclusive to *Yponomeuta*, but rather widespread in microlepidoptera: *Monopis*, *Crypsithyrodes*, and *Crypsithyris* (Tineidae) species are characterised by a hyaline patch in the discal cell of the forewing (Lee et al., 2016; Robinson, 1980; Xiao and Li, 2005) (Figure 3.1); and members of the *Tinea pellionella* species complex (Tineidae) possesses a hyaline patch at the base of the forewing, just below the *subcosta* (Robinson, 1979) (Figure 3.1).

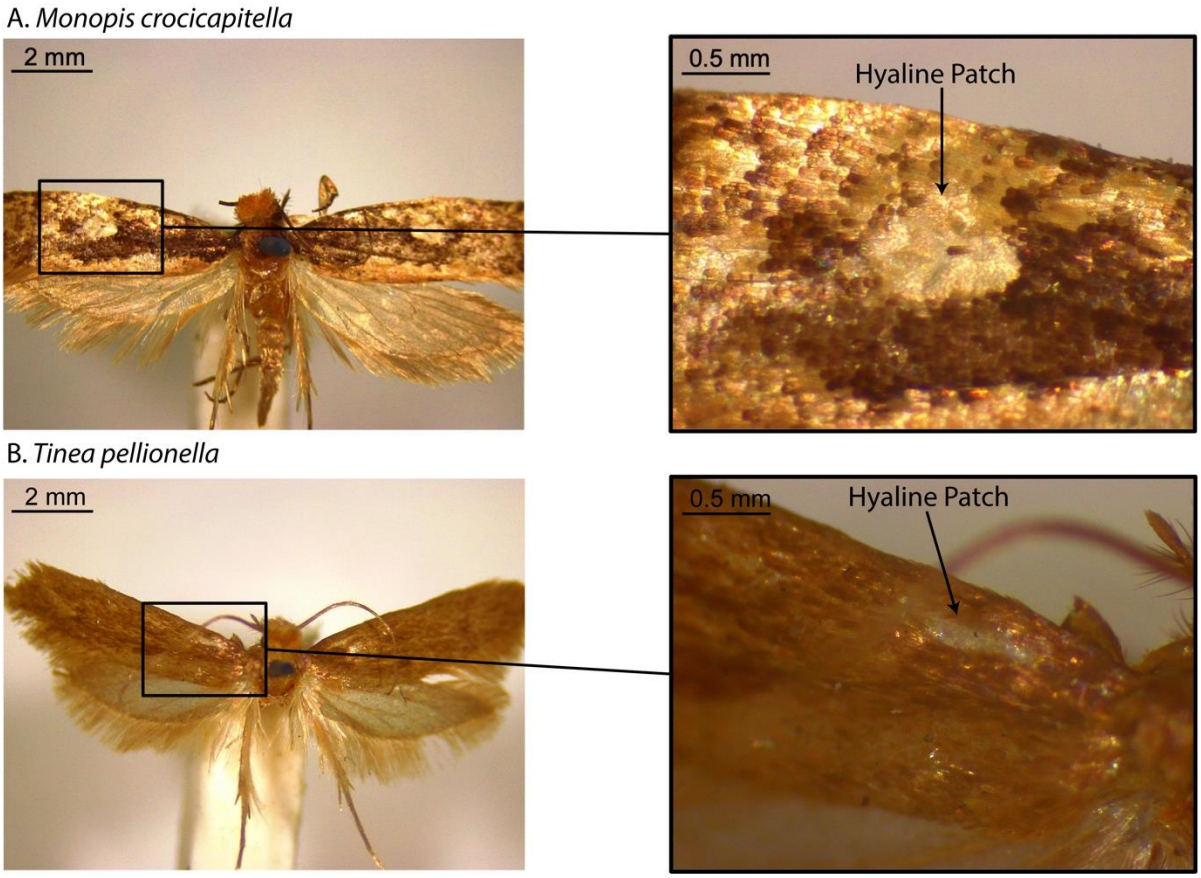


Figure 3.1 Tineinae (Tineidae) Hyaline Forewing Patches Typical examples of the hyaline patches found on the forewings of (A) *Monopis* species (in discal cell) (Huang et al., 2011; Lee et al., 2016; Robinson, 1980), and (B) *Tinea* species (below *subcosta*) (Robinson, 1979).

3.2.1 Aims

Generally, hyaline wing patches, such as the above examples, are only documented in the literature if they serve as identification features. Testing the assumption that wingbeat powered sound production by ATs is more widespread, I conduct a comprehensive search for hyaline patches in microlepidoptera. I then use available live specimens to test whether candidate ATs produce ultrasound with acoustic features similar to known anti-bat signals.

This chapter thus has three aims:

1. To determine the phylogenetic spread of candidate wingbeat-powered ATs within the microlepidoptera, with specific focus on the Tineidae (as hyaline patches are already documented in some taxa) and the superfamily Yponomeutoidea (with known sound producer *Yponomeuta*).
2. To test new candidate ATs for in-flight sound production functionality.
3. To evaluate the functionality of any such sounds through comparisons of their acoustic properties with those of known anti-bat sounds, and by relating this with the life histories of the respective microlepidopteran species.

3.3 Methods

3.3.1 Phylogenetic spread of candidate ATs

3.3.1.1 Image analysis

For each of the following three phylogenetic analyses I assessed taxa for the presence of known and candidate ATs. This was primarily achieved by looking through online image databases of microlepidoptera. The majority of photographs were assessed from the Barcode of Life Database (BOLD) (Ratnasingham and Hebert, 2007), but the website jpmoth.org (Gamania and Uuugi, n.d.) was also used for the Yponomeutoidea analysis, as was microscopic assessment of specimens from the Bristol Museum and Art Gallery and the Natural History Museum, London.

A known AT was defined as a hyaline patch in the same position on the wing as related taxa known to produce wingbeat-powered sound, e.g. a hindwing hyaline patch in a member of the Yponomeutinae. A candidate AT was defined as a hyaline patch on the wing with no obvious other function such as visual patterning. If possible, for every species suspected of possessing an AT, multiple specimens were assessed to confirm the presence of the structure. This helped to prevent false positives due to symmetrical specimen damage.

3.3.1.2 Candidate ATs in the microlepidoptera

This comprehensive assessment of the presence of ATs concerns all microlepidopteran taxa included in a recent molecular phylogeny of the Lepidoptera (Regier et al., 2013) (276 taxa, mostly to genus level), up to and including the Gelechioidea superfamily. Additionally, for all subfamilies containing AT-possessing taxa, all genera on BOLD Systems were also assessed. Despite the Pyraloidea being considered microlepidoptera due to their size, they were excluded from this analysis. These moths are not likely to

possess ATs because they already possess an anti-bat defence (hearing), and although sound production can be an effective defence for tympanate moths, wingbeat powered sound production would be counterproductive as its constant nature would excite and habituate their hearing structures. Images of the taxa (generally genus level) used to construct the phylogeny were assessed for the presence of known/candidate ATs.

The original tree from Figure S1 of (Regier et al., 2013) was simplified for this chapter. Likely points of independent evolution of ATs within the microlepidoptera were labelled on the tree. During the searches the Tineidae genus *Monopis* and the Cosmopterigidae subfamily Scaeosophinae were found to possess candidate ATs. These taxa were not included in the original phylogeny (Regier et al., 2013), so they were added to this analysis.

3.3.1.3 Candidate ATs in the Tineidae

Photographs of 170 species (102 genera, 15 subfamilies) of Tineidae were assessed. Initially one species of each genus on BOLD was assessed; if a structure was discovered, all other species of that genus were assessed. Between one and twenty photographs (individuals) were assessed per species for the presence of hyaline patches on either the forewing or hindwing. In the case of the genus *Chrypsithyris* (four species) images and species descriptions were used from Xiao and Li (2005) as this genus is not present on BOLD. Images for *Niditinea saboskyi* were used from (Metz et al., 2018).

3.3.1.4 Phylogenetic analysis

As far as I am aware, a detailed molecular phylogeny of the Tineidae does not exist. Thus, one was created to add a phylogenetic reference to my data, on which I plotted the occurrence and type of candidate ATs. The tree was created, under my instruction, by Brogan Harris of the Paleobotany group at the University of Bristol.

Using publicly available data, a phylogenetic tree of the Tineidae family was created using the Cytochrome Oxidase Subunit 1 5’ Region amino acid sequence. *Dolophilodes distinctus* (Philopotamidae: Trichoptera) was used as an outgroup. Excluding the outgroup, 90 species from 19 genera in the family Tineidae were included in the analysis.

Protein sequences were downloaded from BOLD and the NCBI. Homologous sequences were aligned using MAFFT version 7. Alignments were then trimmed using BMGE 4.0 using a BLOSUM62 matrix. The alignment was visualised and manually

checked using SEAVIEW. Phylogenetic trees were constructed using IQ-Tree. Model fitting for the LG mixture C* mixture model was undertaken (* Model fit for C10 through to C60). The best fitting model was also run in IQ-Tree with 10000 ultrafast bootstraps. Trees were created using ITOL. See Table 3.1 for references.

Table 3.1 Resources used in the creation of the Tineidae phylogeny.

Resource	Source	Web Link
Data	NCBI	https://www.ncbi.gov/protein/
Data	BOLD Systems	http://www.boldsystems.org/
MAFFT	Katoh *et al*., 2002	https://mafft.cbrc.jp/alignments/software
BMGE	Criscuolo & Gribaldo, 2010	http://gensoft.pasteur.fr.docs/BMGE/1.0/BMGE_doc.pdf
IQ-Tree	Nguyen *et al*., 2015	http://www.iqtree.org/
LG Model	Le & Gascuel, 2008	http://www.atgc-montpellier.fr/models/index.php?model=lg
ITOL	Letunic & Bork, 2007	http://itol.embl.de/

3.3.1.5 Candidate ATs in Yponomeutoidea

In order to discover the phylogenetic spread of *Yponomeuta* ATs I assessed as many moths as possible from the Yponomeutoidea superfamily, using either light microscopy or analysis of online images from BOLD or jpmoths.org (Gamania and Uuugi, n.d.; Ratnasingham and Hebert, 2007) to determine the presence of hindwing hyaline patches. Due to the limited resolution of the images from the two online sources the presence of the typical striated band was not usually detectable, so instead all specimens were assessed for the presence of the hyaline patch alone. However, David Agassiz has confirmed that every yponomeutid species he has investigated possessing a hyaline patch also possesses the striated band (Agassiz, D. Personal Communication, Oct 2017). The results were then compared to a recent molecular phylogeny of the Yponomeutoidea (Sohn et al., 2013) to determine the phylogenetic distribution of the structure.

The poor fidelity of the online images meant that a diagnostic of the presence or absence of the structure could not be attained unambiguously in all cases. Additionally, I did not have access to all species included in the phylogeny. Therefore six terms were used to classify the species present on the phylogenetic tree: (1) hyaline patch present, (2) hyaline patch absent, (3) hyaline patch likely present (used when the structure appeared to be present but was partially obscured by wings or light), (4) hyaline patch likely

absent (used when the structure appeared to be absent, i.e. scales were present in the region of interest, but it was partially obscured by wings or light), (5) hyaline patch present in other species of the genus, and (6) hyaline patch absent in other species of the genus. Terms (5) and (6) could be used in conjunction with each other, due to occasional intrageneric differences.

3.3.2 Sound production by candidate ATs

3.3.2.1 Insect selection and collection

I selected moths based on availability; specimens of two Tineinae species possessing hyaline patches (*Monopis crocicapitella* n=2, and *Tinea pellionella* n=7), one Tineinae species lacking patches (*Tineola bisselliella* n=6), one Oecophoridae species possessing patches (*Endrosis sarcitrella* n=4) and one lacking them (*Hofmannophila pseudospretella* n=2) were tested for sound production. All *T. pellionella* specimens were wild caught from three locations within Bristol, UK where the moths were caught within houses, all *T. bisselliella* specimens were taken from a wicker basket left on a street in Bristol, UK, both *M. crocicapitella* specimens were caught at one location in Weston-Super-Mare, UK using a mercury vapour moth trap in a suburban garden, and all *E. sarcitrella* and *H. pseudospretella* were caught from two houses within Bristol, UK. Moths were either flown immediately or kept in a refrigerator between 4-6°C for up to 24 hours before being flown. Keeping insects at this temperature increases their longevity.

3.3.2.2 Tethering method

Moths were first recorded in free flight, if they did not produce sound, they were no longer used, if they did then they were subsequently tethered. Due to the small size of the insects, I used a similar tethering method to O'Reilly et al. (2019) (see Chapter 2 Figure 2.3 b); I inserted a 0.14 mm diameter insect pin into the dorsal meso/prothorax until the tip just punctured the ventral side. Similarly to the moths tested in O'Reilly et al. (2019) I found that all test specimens flew for prolonged periods post tethering, so I continued with this as my tethering method.

For audio recordings, pinheads were inserted into modelling clay attached to a flexible arm (Manfrotto + Co. Spa, Cassola, Italy), which allowed me to reposition the moth. I positioned the moth upside down as it elicited more prolonged flight compared to

normal orientation. This stronger flight is probably due to the unusual gravitational pull on the insect causing it to try and return itself to its natural flight orientation.

3.3.2.3 Audio recordings

All audio recordings (16bit, sampling rate 500 kHz) were made using USG Omnidirectional Electret Ultrasound Knowles FG-O microphones connected to an UltraSoundGate 1216H^{200} recorder, run through Avisoft Recorder USGH software (all Avisoft Bioacsoutics, Berlin, Germany). For the frequency response of the microphone used here see Appendix 4. All recordings were made in a semi-anechoic chamber (Industrial Acoustics Company Ltd., Winchester, UK) to reduce reverberation.

Individual moths were initially placed in a 24x24x24" BugDorm-1 Insect Rearing Cage (Megaview Science Co., Ltd., Taichung City, Taiwan) with one microphone positioned through a central sleeved hole on one side of the cage. Flight was initiated through tactile stimulation of the moth, flicking or tapping the cage where the insect was at rest. These free-flight recordings were initially analysed for the presence of any acoustic signal. If sound production was discovered, tethered recordings were subsequently made. For tethered recordings the insect was positioned 30-50 mm from a microphone oriented perpendicular to the centre of the lateral axis of the moth similarly to 2018 experiments with *Yponomeuta* in Chapter 2 (see Figure 2.3b). To reliably initiate flight, tethered moths were first given a small (~5 mm diameter) ball of paper or foam to hold, this was removed when flight was required.

3.3.2.4 Hearing tests

Prior to free-flight recordings, once a moth was released into the cage it was exposed to an ultrasonic stimulus known to elicit the anti-bat behaviours of moths with hearing capabilities (Juliana et al., 2007) at a distance of around one metre from the centre of the cage. The moth was exposed to the stimulus both at rest and during flight, and its behaviour observed. A Dazer II Ultrasonic Dog Deterrent (Dazer International, London, UK) was used as the stimulus; it produces a 25 kHz tone at 118.1 dB SPL (at 0.1 m). Reactions were defined as a sudden cessation of flight, or any other typical anti-bat escape/avoidance manoeuvre (Miller and Surlykke, 2001), or twitching, commencement of flight, or dropping from its perch if the moth was at rest.

If multiple individuals of the same species were caught on the same day, they were placed in the BugDorm-1 together and their behaviour in response to flight, and

therefore sound production, of other individuals was observed. This was possible once each for *M. crocicapitella* (two individuals), *T. pellionella* (two individuals), and *E. sarcitrella* (two individuals).

3.3.2.5 Ablation experiments

Ablation was attempted on all individuals of *M. crocicapitella, T. pellionella*, and *E. sarcitrella*, however, due to the small size of the moths and their hyaline patches, this proved difficult. In all but one individual of each of the two Tineinae species the ablation attempt resulted in enough damage to the wings to render them unable to fly. Therefore, ablation results were only taken from one individual of each Tineinae species. *E. sarcitrella* patches were more fragile than those of the Tineinae, therefore a cruder method of ablation (removal of the hindwings) was initially used to confirm the general location of the sound producer (n=1). More specific ablation attempts similar to the Tineinae, were unsuccessful in the three remaining *E. sarcitrella* individuals. For the successful ablations of *M. crocicapitella* and *T. pellionella*, recordings were made from two treatments for each moth, firstly the right hyaline patch was ablated, and secondly the left hyaline patch was ablated.

Tineinae ablation was achieved using a size 0.14 mm diameter insect pin under a 50x magnification dissection microscope (Leica EZ5 Stereo Microscope, Leica Microsystems, Wetzlar, Germany). Moths were anaesthetised using CO_2 and secured to foam by placing two insect pins in a cross over (not penetrating) both the abdomen and head of the insect, as well as individual pins over the fore and hindwings to hold them extended from the body, thus exposing the hyaline patches. The patch was then punctured with an insect pin and the membrane removed using fine forceps and microdissection scissors. All pins were removed, and the insect was positioned within the recording set-up, holding a small piece of paper or foam. It was left for between 15 and 120 min to recover and checked every 15 min for pre-ablation flight behaviour, and then post-ablation recordings were made.

3.3.2.6 Acoustic analysis

I analysed all acoustic recordings using Avisoft SASLab Pro (version 5.2.07, Avisoft Bioacoustics, Berlin, Germany). Sounds were analysed for source level, peak frequency, high and low frequency (bandwidth), click detection distance, shorter click burst click duration, longer click burst click duration, duty cycle, number of clicks per burst, and

number of clicks per wingbeat. For every analysis I chose to use 10 consecutive wingbeats from a good flight period (judged to be a set of consistently high amplitude click bursts) for each individual.

Acoustic characteristics were determined following the methods outlined for *Yponomeuta* spp. in Chapter 2. Detection distance by a bat of the clicks of *M. crocicapitella*, *T. pellionella*, and *E. sarcitrella* was also calculated as per the methods outlined in Chapter 2. The principal difference was that here I did not know which click bursts were the upper and lower burst, so where clicks needed to be taken from the upper burst for analysis, I took clicks from the longer burst (the burst lasting the longest amount of time).

3.3.2.7 *Ethmia* acoustics

Under my instruction, a collaborator on my previous work on *Yponomeuta* acoustics, Dr David Agassiz (O'Reilly et al., 2019) took a USB ultrasonic microphone (Ultramic250K, Dodotronic, Italy) to record AT-possessing moths in Kenya. He captured and recorded one *Ethmia bicolorella* (Ethmiidae) in free flight (16bit, sampling rate 250 kHz).

3.4 Results

3.4.1 Phylogenetics

3.4.1.1 Phylogenetic spread of ATs in the microlepidoptera

276 taxa from the most basal microlepidoptera through to the Gelechioidea superfamily were assessed for the presence of candidate ATs (hyaline patches). The results were plotted on a simplified version of a recent lepidopteran phylogeny (Regier et al., 2013) (Figure 3.2). The distribution of candidate ATs is best explained assuming 15 independent evolutionary origins (Figure 3.2).

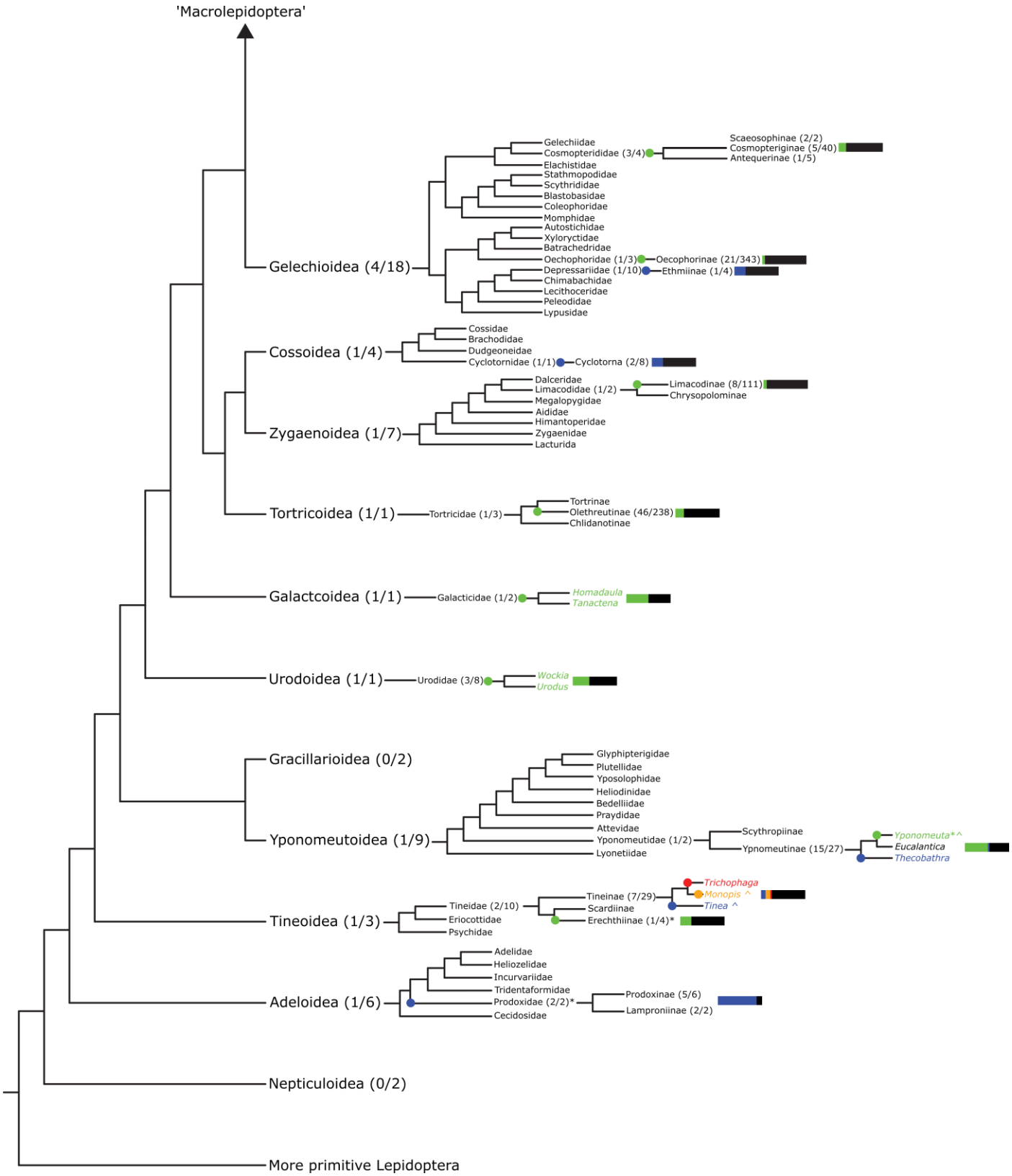


Figure 3.1 Phylogeny of the 'Microlepidoptera' (here defined as taxa below and including the superfamily Gelechioidea) adapted from (Regier et al., 2013). The spread of aeroelastic tymbals (ATs) is represented at various taxonomic levels, beginning with superfamilies and ending in genera. For each taxonomic level, the fraction of subtaxa possessing ATs is given in parentheses. Following superfamily, if ATs are present, all families are presented, and then only relevant subfamilies (i.e. possessing ATs or showing evolutionary relationships). In subfamilies with multiple types of AT (see Figure 3.3), a genus tree is presented to show evolutionary relationships. Coloured and black bars represent the ratio of genera possessing ATs within the highest taxonomic rank containing ATs. Colours correspond to the location of the AT on the wing and match Figure 3.3, and black represents no obviously detectable structure. Coloured nodes represent the most likely point of evolution of ATs, and colours again correspond to the locations in Figure 3.3.

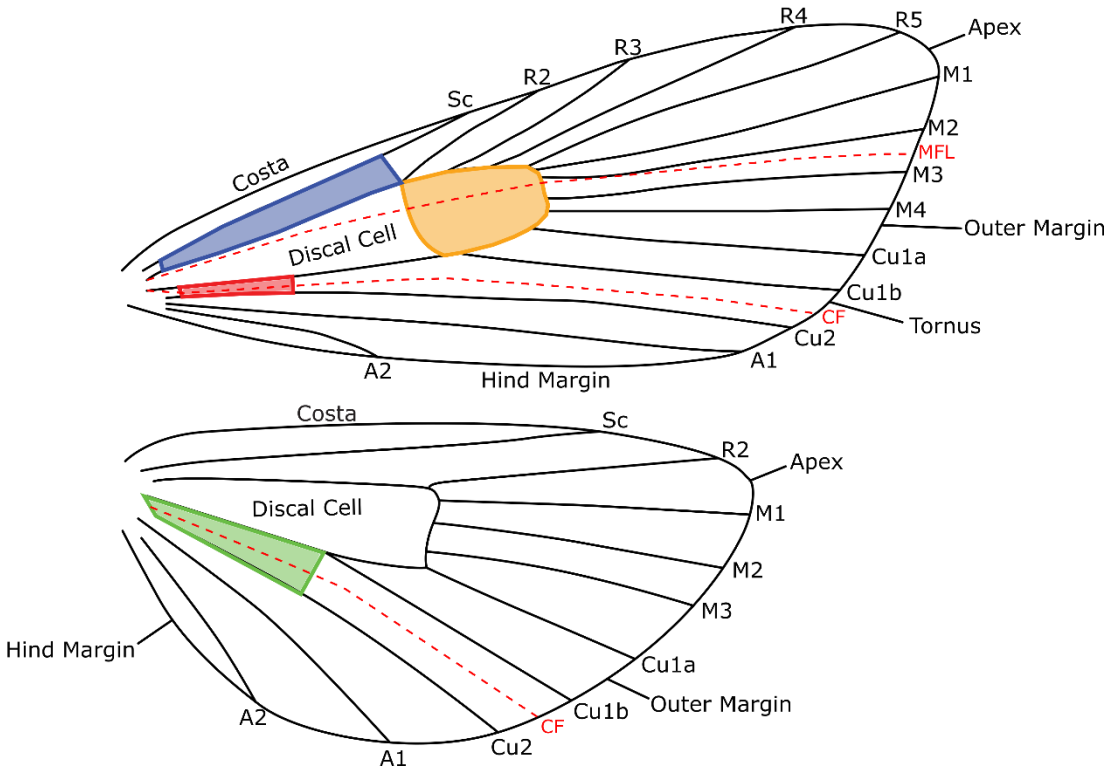


Figure 3.3 Microlepidopteran aeroelastic tymbals. Locations of aeroelastic tymbals (ATs) on microlepidopteran wings (modified from Watson and Dallwitz, 2003 onwards). Shaded areas show locations of ATs, and dashed red lines represent flexion lines in the wing, the median flexion line (MFL) and the claval furrow (CF). Vein labelling: Sc (Subcosta), R (Radial), M (Medial), Cu (Cubital), and A (Anal), followed by vein number.

ATs have been identified in four different locations on microlepidopteran wings (Figure 3.3): (1) at the forewing base between the subcostal and radial veins in the cell directly above the discal cell (evolved five times, blue), (2) directly within the apex of the discal cell itself (evolved once, orange), (3) in the cell directly below the discal cell (evolved once, red) and (4) at the base of the hindwing in the cell directly below the discal cell (evolved nine times, e.g. Yponomeutinae, green).

3.4.1.2 Phylogenetic spread of ATs in the Tineidae

Using the purpose made Tineidae phylogeny, analysis of 174 species from 103 genera in 15 subfamilies of the Tineidae revealed that hyaline patches, likely to be ATs, were present in 46 species in seven genera, all within the subfamily Tineinae. These structures can be grouped as *Tinea*-like ATs (TATs, hyaline patches like those of *T. pellionella*, Figure 3.1), *Monopis*-like ATs (MATs, hyaline patches like those of *Monopis*, Figure 3.1) and *Trichophaga*-like ATs (TrATs, Figure 3.4). Additionally, the previous analysis using the (Regier et al., 2013) phylogeny revealed *Yponomeuta*-like structures in the Erechthiinae genus *Erecthias*. This genus was not, however, included in this phylogenetic analysis.

TATs are present in nine of 38 species of *Tinea* as well as one of two *Praeacedes* and four of five *Niditinea* species examined, including the newly discovered species *N. sabroskyi* (Metz et al., 2018). MATs are present in all *Monopis* analysed (24 species) as well as the genera *Crypsithyrodes* (one species) and *Crypsithyris* (four species), *Tinea unomaculella* possesses a light spot in the same area but I believe this is colouration not a tymbal. TrATs are small (~1 mm in length) hyaline patches near the base of the forewing, likely between veins Cu_{1b} and Cu_2 (Figure 3.3 and Figure 3.4), and are limited to the genus *Trichophaga*, present in at least three of the six species analysed.

TATs vary in size, with the relatively large, conspicuous examples being found in *T. steueri* and *T. svenssoni*, whereas species such as *T. dubiella* possess much smaller structures. MATs can vary in their size, shape (relatively round to elongated), and their location on the wing in terms of their position along the wing tip to base axis. Nevertheless, the structures always appear to be situated within the discal cell of the forewing and their position is likely due to differences in the length of this cell.

A detailed phylogeny of the Tineidae family does not exist in the literature, let alone one for the subfamily Tineinae, therefore using publicly available genetic data a maximum likelihood tree was created from the Cytochrome Oxidase Subunit 1 5' Region amino acid sequence. Although this did not provide the most robust tree, it at least provided a phylogenetic reference for this project. The maximum likelihood tree (Figure 3.4), groups all bar one species of *Monopis* as a single clade, with the three *Trichophaga* species forming a sister clade to this. *Monopis fenestratella* possesses a MAT but is placed away from the rest of the genus within the main *Tinea* clade. The TAT-possessing species do not form a single clade but are instead split into two main clades with one species, *T. trinotella*, placed away from these two groups. The first of these two clades exclusively contains *Niditinea* and *Praeacedes* species, and the second exclusively *Tinea* species. The second, with the addition of *T. columbariella* and *T. niveocapitella*, consists of the already established *Tinea pellionella* species complex (Robinson, 1979). *Tineola bisselliella* (no hyaline patch) is placed as the only non-*Tinea* species in a clade containing mostly TAT-possessing species.

3.4.1.3 Phylogenetic spread of ATs in the Yponomeutoidea

Of the 229 species assessed from the Yponomeutoidea superfamily, at least 49 possess an AT; the true number is probably higher as the background colour of photos and the colour the moths' hindwings prevented definitive identification of the structure in several species. All bar one genus possessing a hyaline patch were within the Yponomeutinae subfamily of Yponomeutidae (Figure 3.5), the exception being *Ochsenheimeria* (Ypsolophidae). *Ochsenheimeria urella* possesses a hyaline hindwing region encompassing what is the hyaline cell in the Yponomeutinae.

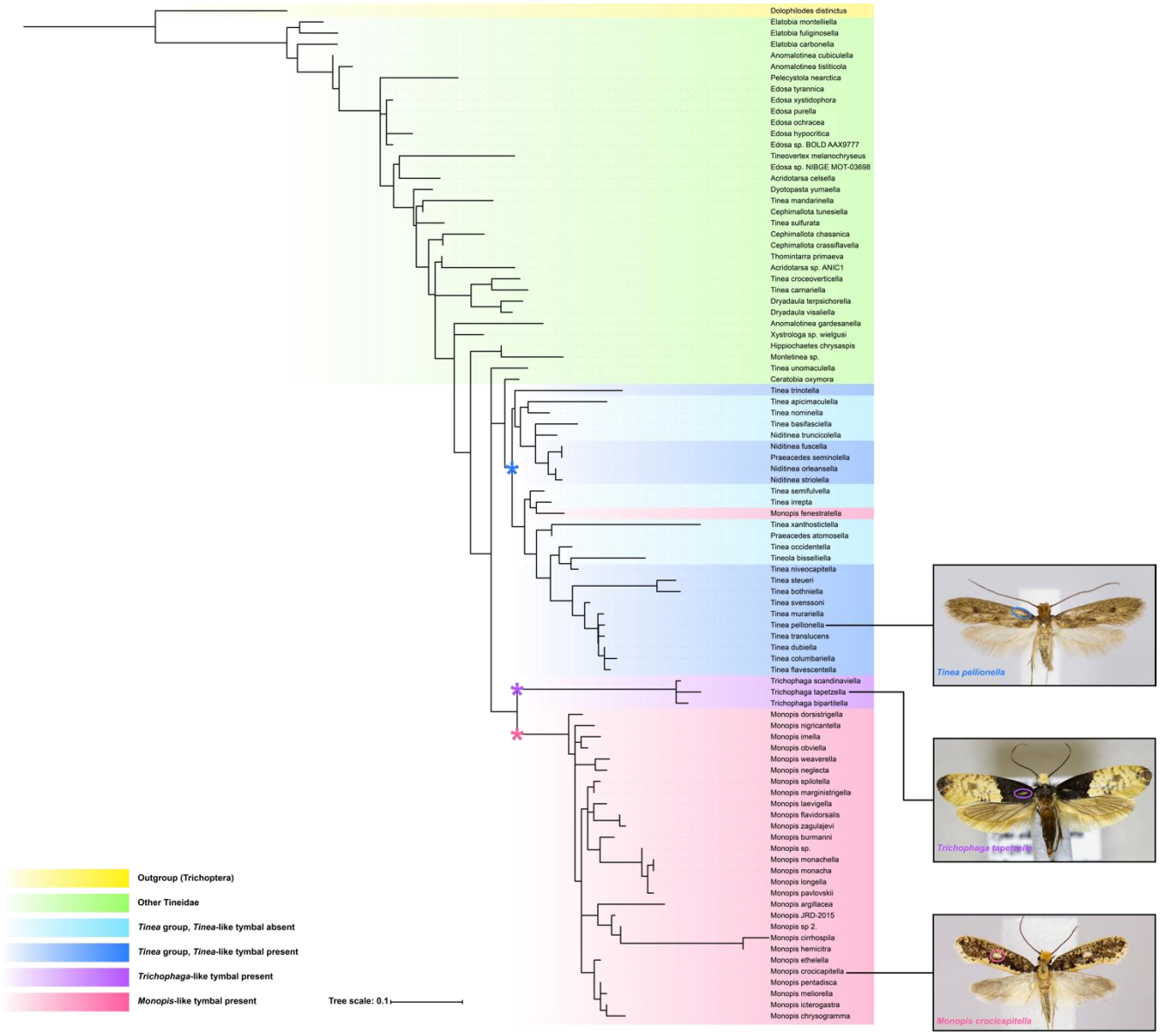


Figure 3.4 Phylogenetic tree of the Tineidae Cladogram of a maximum likelihood tree created from a Cytochrome Oxidase Subunit 1 5' Region amino acid sequence. The three Tineinae ATs (*Tinea*-like, *Monopis*-like, and *Trichophaga*-like) are labelled on an example species of each. Coloured asterisks indicate likely origins of the three ATs.

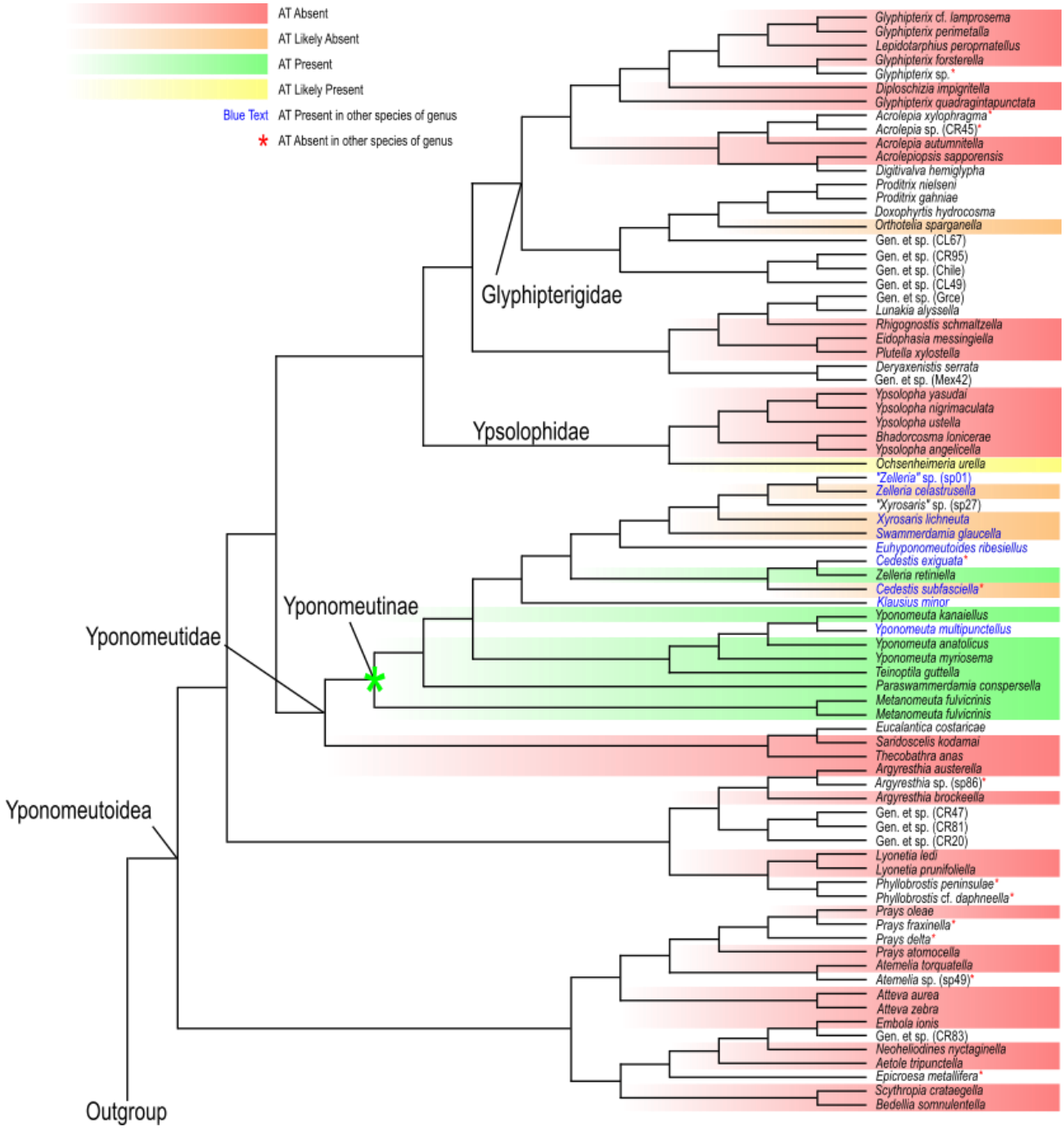


Figure 3.5 Phylogenetic tree of the Yponomeutoidea superfamily adapted from (Sohn *et al.* 2013). The presence of hindwing hyaline patches between the Cu_{1b} and Cu_2 veins is indicated by colour code, and the likely point of aeroelastic tymbal (AT) evolution is indicated by a green asterisk.

3.4.2 **Acoustics**

3.4.2.1 Tineinae and Oecophoridae hyaline patches are sound producing structures

Live specimens from three Tineinae and two Oecophoridae species were available for acoustic testing. All three species possessing hyaline patches, *Monopis crocicapitella* (forewing patch), *Tinea pellionella* (forewing patch), and *Endrosis sarcitrella* (*Yponomeuta*-like hindwing patch), produced two bursts of broadband ultrasonic clicks

with every wingbeat (Figure 3.6). Although high-speed video was not used to confirm this, the clicks are acoustically similar to the in-flight clicks produced by the ATs of *Yponomeuta* species (Yponomeutidae) (O'Reilly et al., 2019) in that they show a bimodal regularity (likely two different bursts per wingbeat, one on the up and one on the downstroke), they exclusively occur during flight, and the two bursts differ in length (Figure 3.6).

Both species lacking hyaline patches, *Tineola bisselliella* (webbing clothes moth) and *Hofmannophila pseudospretella* (brown house moth), did not produce any acoustic emissions during flight. Although males of *T. bisselliella* are known to produce low frequency substrate-borne sounds (Takács et al., 2003), I was not attempting to record these and the recording set-up was not designed to do so, i.e. the frequency response of the recording microphones was not optimised for these sounds, and I was not attempting to record substrate-borne vibrations.

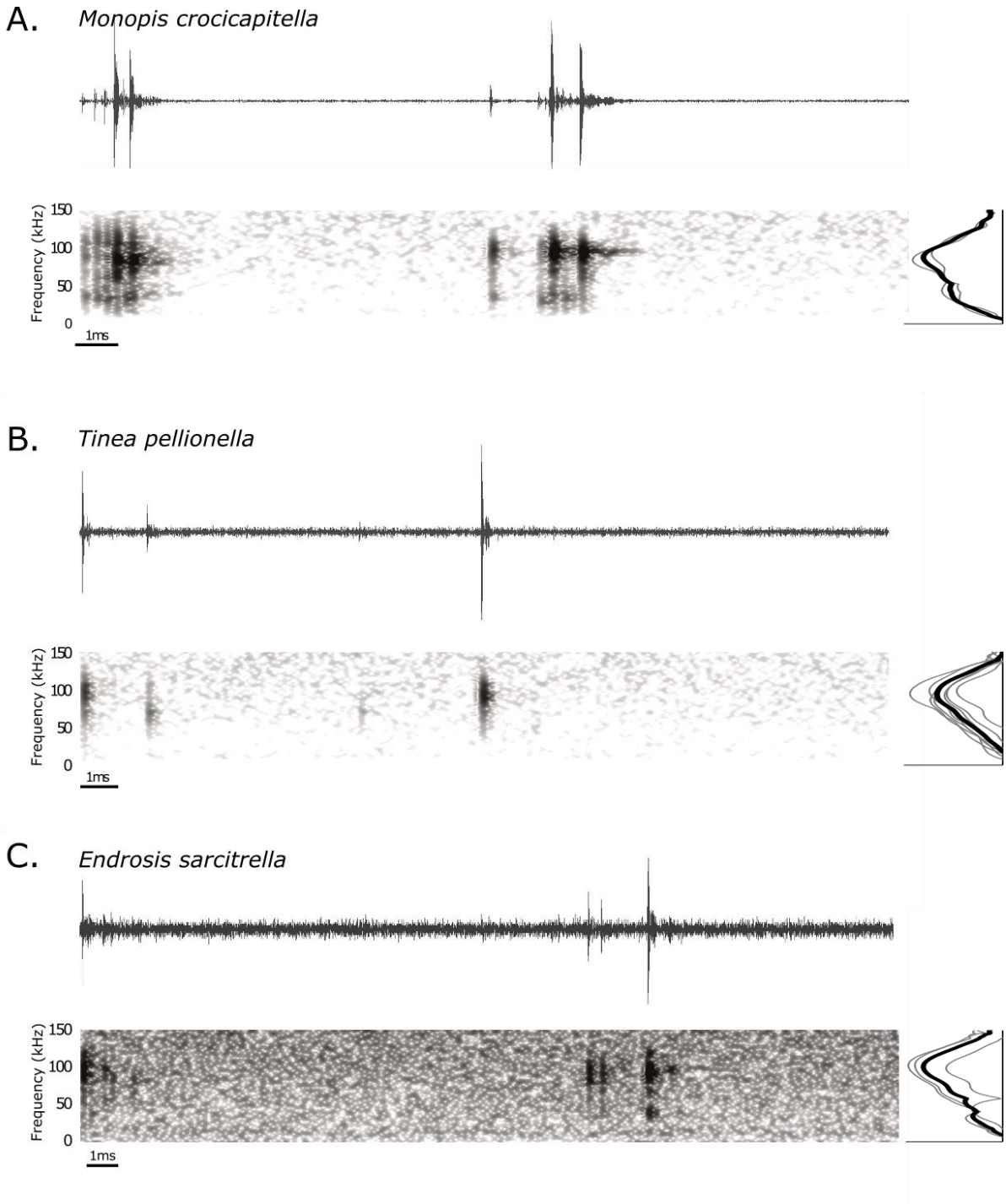


Figure 3.6 Spectral and temporal characteristics of troglophilic microlepidoptera sounds The waveform and spectrogram (FFT size 512, window FlatTop, overlap 75%) of typical examples of in-flight acoustic emissions of three species of cave-dwelling moths, (A) *Monopis crocicapitella* (Tineidae), (B) *Tinea pellionella* (Tineidae), and (C) *Endrosis sarcitrella* (Oecophoridae). Each panel represents one full wingbeat showing the two bursts of clicks produced with each wingbeat cycle, beginning with the first click of the burst with the shortest inter-click interval, and ending immediately prior to the first click of the next equivalent burst. To the right of each spectrogram is a power spectrum showing the normalised click amplitude for the species mean (black line) and individuals (grey lines, for each species n see methods). Time scales vary between plots, and spectrograms are not calibrated for amplitude.

Successful ablation of the hyaline patches of both *M. crocicapitella* and *T. pellionella* (n=1 for both species) eliminated sound production, whilst ablation of one hyaline patch, leaving the other intact, effectively halved the number of clicks produced per wingbeat, 22.95 ± 3.4 and 3.8 ±0.9 pre ablation, and 12.9 ±1.1 and 2.2 ±0.4 post ablation (mean ± SD) for *M. crocicapitella* and *T. pellionella* respectively. Removal of both *E. sarcitrella* hindwings eliminated sound production.

3.4.2.2 *Ethmia* produce sounds during flight

Under my instruction, Dr David Agassiz recorded one individual *Ethmia bicolorella* in free-flight in Kenya. The moth produced bursts of ultrasonic clicks during flight characteristic of AT sound production (Figure 3.7).

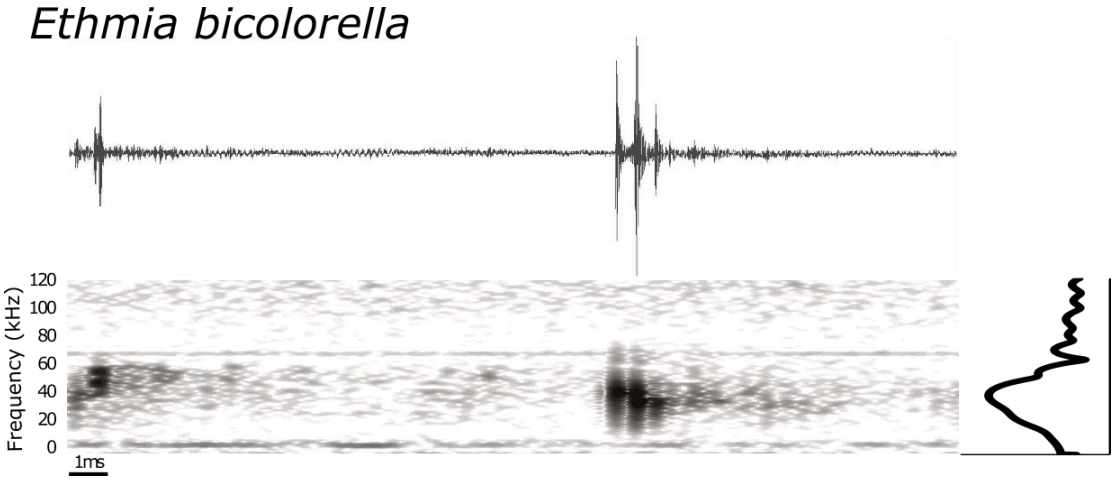


Figure 3.7 Spectral and temporal characteristics of *Ethmia bicolorella* sounds The waveform and spectrogram (FFT size 512, window FlatTop, overlap 75%, not calibrated for amplitude) of the in-flight acoustic emissions of *Ethmia bicolorella* (Depressariidae). One full wingbeat is represented, showing the two bursts of clicks produced. The panel begins with the first click of the burst with the shortest inter-click interval and ending immediately prior to the first click of the next equivalent burst. To the right of the spectrogram is a power spectrum showing the normalised amplitude of the loudest click recorded from this individual.

3.4.2.3 Acoustic characterisation of AT sounds

I analysed bursts and individual clicks from *M. crocicapitella*, *T. pellionella*, and *E. sarcitrella* for amplitude, spectral, temporal, and duration information. In addition, I calculated the distance at which bats could detect these clicks (Table 3.2). All three species produce relatively loud (64.6, 56.9, and 54.0 dB peSPL respectively) ultrasonic, broadband clicks (41.2-111.7, 54.3-125.1, and 45.8-128.9 kHz respectively) with high peak frequencies (88.1, 92.1, and 100.0 kHz respectively).

The sounds of all three moths fall within the known frequency range of anti-bat sounds of the Arctiinae, Sphingidae and Geometridae (Barber and Kawahara, 2013; Corcoran et al., 2010; Corcoran and Hristov, 2014). Their low duty cycles (Table 3.2) are also similar to some of the aposematic signalling Arctiinae such as *Cosmosoma stibasticta* and *Amplicincia* near *mixta* (Corcoran et al., 2010).

Table 3.2 Acoustic properties of three cave-dwelling microlepidoptera species Acoustic properties (mean ± SD; n= clicks) of the clicks of *Tinea pellionella* (Tineidae, seven individuals), *Monopis crocicapitella* (Tineidae, two individuals), and *Endrosis sarcitrella* (Oecophoridae, four individuals). For each of 10 consecutive wingbeat cycles the highest amplitude click was selected. From its waveform the source level, and from the logarithmic spectrum (Hamming) peak, low and high (highest and lowest frequency 15 dB below the amplitude of the peak frequency) frequencies were measured. Click detection distance was calculated from source levels and peak frequencies using an adaptation of the sonar equation, including frequency dependent attenuation (see methods). Ten wingbeat cycles (20 bursts) were analysed for click duration, duty cycle and the number of clicks per burst (see methods). Sp =Species, F=Family, SL=Source Level (dB peSPL 0.1m), PF=Peak Frequency (kHz), LF=Low Frequency (kHz), HF=High Frequency (kHz), DD=Click Detection Distance (m), SCD=Shorter Burst Click Duration (µs), LCD=Longer Burst Click Duration (µs), DC=Duty Cycle (%), NHMC=Number of Clicks per Half Modulation Cycle (Burst), NMC=Number of Clicks per Modulation Cycle (Wingbeat).

Sp.	F	SL	PF	LF	HF	DD	SCD	LCD	DC	NHMC	NMC
Tinea pellionella	Tineidae	56.9 ± 0.7 (n=70)	92.1 ± 3.7 (n=70)	54.3 ± 3.1 (n=70)	125.1 ± 4.8 (n=70)	4.1 ± 0.2 (n=70)	13.9 ± 1.0 (n=70)	13.9 ± 1.3 (n=70)	0.4 ± 0.2 (n=7)	3.8 ± 0.9 (n=140)	7.6 ± 1.0 (n=70)
Monopis crocicapitella	Tineidae	64.6 ± 2.0 (n=20)	88.1 ± 3.1 (n=20)	41.2 ± 8.0 (n=20)	111.7 ± 4.3 (n=20)	5.9 ± 0.4 (n=20)	14.5 ± 1.5 (n=20)	13.8 ± 0.8 (n=20)	0.8 ± 0.04 (n=2)	11.2 ± 2.0 (n=40)	22.95 ± 3.4 (n=20)
Endrosis sarcitrella	Oecophoridae	54.0 ± 1.1 (n=40)	100.0 ± 1.5 (n=40)	45.8 ± 2.3 (n=40)	128.9 ± 3.6 (n=40)	3.4 ± 0.1 (n=40)	9.7 ± 1.4 (n=40)	13.6 ± 0.9 (n=40)	0.2 ± 0.1 (n=4)	4.5 ± 2.5 (n=80)	9.1 ± 1.9 (n=40)

3.4.3.1 Hearing tests

All individuals (excluding *E. bicolorella*) were exposed to a sound source known to elicit the anti-bat behaviours of eared insects (Juliana et al., 2007). No individual of any species showed any reaction, such as cessation or initiation of flight, sudden movement, or any change in flight direction. Additionally, when the insects were obtained as groups of two or more individuals, they were housed together and no individual was observed reacting to flight, and therefore sound production, of the other (n=2 for all species).

3.5 Discussion

3.5.1 Phylogenetic spread of ATs in the microlepidoptera

ATs are a widespread trait within the microlepidoptera having independently evolved 15 times (Figure 3.2) if all 10 candidate structures are confirmed to be sound producers (Figure 3.2). Even if none of the candidate structures function as ATs, there are still five independent examples of the evolution of this structure within the microlepidoptera (Yponomeutinae, TATs, MATs, *E. sarcitrella*, and *Ethmia*), which itself is an incredible example of convergent evolution. As this was not, and could never be, an exhaustive assessment of the microlepidoptera, there are more than likely many more examples still undiscovered.

The positioning on the wing of ATs may provide some insight into how they function. Interestingly, all four AT locations place them near flexion lines (Figure 3.3). Flexion lines are lines along which an insect wing can show flexibility during the wingbeat. The claval furrow is a flexion line found in most insect wings, and the median flexion line is found in the forewing (and occasionally hindwing) of many insect taxa, and usually runs between the medial and radial veins (Dudley, 2000).

The hindwing claval furrow appears to play a role in *Yponomeuta* AT actuation (O'Reilly et al., 2019) (see Chapter 2), and thus it can be presumed to be of similar importance in other taxa with similarly placed ATs. *Trichophaga* ATs are located analogously to *Yponomeuta* ATs but in the fore- not hindwing, and thus, if these are sound producers, the claval furrow is again likely to play a role in actuation.

The median flexion line is not always present in insect wings, and its position on the wing when present can vary between taxa (Wootton, 1979); however, its normal

location transects the discal cell and therefore MATs. Additionally, TATs and similarly positioned ATs are in close proximity to the median flexion line if it passes through the discal cell, but there is also the possibility that in these taxa it may be situated even closer to the tymbal. It is therefore reasonable to predict that MAT and TAT actuation is facilitated by this flexion line. This apparent importance of flexion lines in AT location, and probably in actuation, provides a starting point for further searches for these structures, as well as an important factor in potential modelling of these novel sound-producing systems.

Prior to this study, I briefly assessed the macrolepidoptera for the presence of ATs. Other than the known tymbals of *Amyna natalis*, which are used for sexual communication and are not perpetually active during wing movement (Heller and Achmann, 1993), there were no obvious AT candidates in this suborder. This exclusivity to and convergence within the microlepidoptera of ATs suggests that something about their wings gives them a propensity to be sound producers. Therefore, differences between macro and microlepidoptera wing structure would be another important area of investigation for research into modelling the structure. The most obvious difference between macro- and microlepidoptera is their size. In my opinion, this is the most likely morphological factor that may provide microlepidopteran wings with a propensity to function as sound producers. I believe that wing cell size may be important, as the smaller spaces between wing veins may allow for appropriately sized tymbals in micro- but not macrolepidoptera. Tymbal size is likely to be a factor in determining click characteristics such as frequency; thus, perhaps the cell sizes in macrolepidopteran wings would not allow for ATs that produce clicks with frequencies appropriate for anti-bat sound production.

In assessing the spread of ATs it became apparent that ermine colouration (black/dark spots on a white/light background) is relatively common in AT-possessing taxa. *Yponomeuta* are named for it (small ermine moths), and it is common in *Ethmia* and *Scaeosopha* spp. amongst others. In total it was observed in five AT-possessing groups. Such convergence, and a lack of sexual dimorphism, suggests that ermine colouration may have a defensive function. Ermine colouration could in fact be a visual aposematic signal, especially if these moths are all acoustically aposematic. An interesting possibility that certainly requires further investigation.

The bat-moth evolutionary arms race is currently an area of great research interest for both sensory ecologists and evolutionary biologists, and yet a huge number of taxa remain underrepresented in the current literature. Microlepidoptera are largely ignored in terms of this topic, and this research proves that this suborder is massively understudied. The incredible level of convergence in anti-bat sound producing structures is further evidence in support of microlepidoptera being under great selection pressure from bat predation. As a result of this pressure, the array of acoustic defences these moths possess are probably just as complex and diverse as their larger cousins, and they undoubtedly deserve increased research attention.

3.5.2 Cave-dwelling micromoths and bats

Records of troglophilic (cave-dwelling) invertebrates from various cave systems globally indicate that Tineidae are widely present, particularly in tropical and subtropical regions of the Americas as well as the Balkan states and Australia (Barr and Reddell, 1967; Byun et al., 2014; Cokendolpher and Polyak, 1996, 2004; Eberhard et al., 2014; Hamilton-Smith, 1967; Humphreys and Eberhard, 2001; Jakšić, 2017; László, 2004; Pape, 2014; Peck, 1974, 1975; Polak et al., 2012; Robinson, 1980; Silva and Ferreira, 2015; Society, 2017; Trajano, 2000; Turbanov et al., 2016; Wynne et al., 2005; Wynne and Pleytez, 2005). The Tineidae is a cosmopolitan lepidopteran family (Slootmaekers, 2013), and so it is highly probable that tineids will be present in cave systems globally.

Larvae of the subfamily Tineinae feed on animal detritus, resulting in independence from green plants. This independence allows these moths to permanently inhabit environments such as caves. Indeed, at least 11 species of Tineidae (including species of *Monopis*, *Crypsithyrodes*, *Crypsithyris*, *Tinea*, *Niditinea*, and *Praeacedes*) are known to spend their entire lives within caves feeding as larvae on bat guano or the fungi that grow on it (Robinson, 1980). In addition to the Tineidae, *E. sarcitrella* is also found in caves and bat roosts (Centelles Bascuas, 2015; Mosconi, 2011). *E. sarcitrella* is a pest of stored grain, but is known to be able to subsist on guano and other organic matter (Carter, 1984). These moths exist alongside bats, potential predators, feeding on their faeces, putting them at a perpetual risk of predation. It seems counterintuitive for moths to have initially adapted to live on the faeces of their predators, indeed, guanophagy in cave-dwelling microlepidoptera may have originated before bats, with

moths perhaps feeding on bird guano. However, their current association with bats may help to explain the convergence of ATs in cave-dwelling microlepidoptera.

3.5.2.1 Phylogenetic spread of ATs within the Tineidae

ATs have seemingly convergently evolved four times within the Tineidae, in *Erechthias*, *Monopis*, *Tinea*, and *Trichophaga* (Figure 3.3 and Figure 3.4). Four independent evolutionary origins of a trait within one family is an incredible level of convergence; and the three examples (*Monopis, Tinea,* and *Trichophaga*) within one subfamily (Tineinae) is unprecedented in the Lepidoptera, and generally extremely rare in nature.

There are two lines of support for the convergent evolution of anti-bat sound production by ATs within the Tineinae subfamily. Firstly, morphologically the structures are similar in many aspects, but are sufficiently different in shape and position on the wing to suggest multiple evolutionary origins. And secondly, based on their phylogeny, the distinctly separate *Monopis, Trichophaga* and *Tinea*-like clades suggests three points of evolutionary origin for Tineinae ATs (Figure 3.4). The *Tinea*-like clade is interesting as TATs appear in three separate clades within this. It is more likely that a common ancestor of this clade possessed a TAT, and that secondary loss of this trait has occurred multiple times, rather than the trait having evolved in parallel within this clade. The ancestor to this main *Tinea*-like clade, probably had a troglophilic ecology and as a result had evolved an AT. As the clade diverged and certain taxa switched to less troglophilic ecology, the intensive selection pressure of living with bats reduced and the structures were probably lost due to relaxed selection. Despite this reduced predation pressure, the ability to produce anti-bat sound is still advantageous to survival outside of a cave environment, which may explain why it persists in the *Tinea pellionella* species complex despite these moths not having troglophilic ecology.

As far as I am aware anti-bat sound production in the Tineinae is a unique trait within the Lepidoptera, as it has evolved convergently at least twice, possibly three times, within one subfamily. Convergent evolution of bat defences in the Lepidoptera is common, and has occurred in terms of hearing, sound production, and hindwing decoys (Barber et al., 2015; Corcoran and Hristov, 2014; ter Hofstede and Ratcliffe, 2016), but it rarely, if ever, occurs between such closely related taxa as within one subfamily.

3.5.2.2 Evolution of ATs in cave-dwelling taxa

The widespread presence and diversity of hyaline patches within the subfamily Tineinae is indicative of an event having great selection pressure on the group at some point during its evolutionary history, it is likely that *E. sarcitrella* underwent this same pressure. Given the likelihood of their sounds having an anti-bat function, and the ability of these moths to permanently inhabit caves, it is highly likely this event was either the evolution of echolocating bats around 65 mya (Conner and Corcoran, 2012; Jones and Teeling, 2006), or the colonisation of bat caves by these moths.

Guanophagous moths will indiscriminately feed on bird or bat guano, or other animal products (Robinson, 1980). With birds having evolved considerably earlier than bats (Kumar and Hedges, 1998), it is plausible that the ancestral cave-dwelling, guanophagous tineid shared its abode with cave-roosting birds, much like the swiftlets or oilbirds known today. A cave can provide a geographic mating barrier to populations, and as many Tineinae species can spend their entire lives living in caves (Robinson, 1980), ancestral moth populations could have become isolated in caves, leading to speciation. Then, following the evolution of echolocating bats and their colonisation of caves, this strong predation pressure, the geographic isolation, and an apparent propensity for wings to be sound producers resulted in the convergent evolution of ATs in the Tineinae.

3.5.2.3 Humans and AT-possessing taxa

Both Tineinae and *E. sarcitrella* have a close association with human habitation; with the former pests of textiles and other household items (Brokerhof et al., 1993), and the latter a pest of stored grain (Carter, 1984). Both taxa are also frequently found in caves and are capable of subsisting on bat guano. Sound production through wingbeat-powered tymbals therefore appears to be a common trait in moths associated with both caves and human buildings.

Caves and buildings share similar environmental properties; they both offer shelter from the elements and thus provide relatively stable temperatures and humidity (depending on the region of the cave) (Howarth, 1980; Landsberg, 1954). Additionally, both environments tend to provide food sources; guano, the fungi that grow on it, or keratin-rich animal products in the case of caves, and within human buildings there are many keratin-rich objects, for example clothes, carpets, upholstery, and even dead

animal material in the form of skins and bone (frequently used as decoration). A human building is an alternative to a cave in both environmental conditions and food supply for cave-dwelling moths, and this may offer an explanation as to why it appears so many household pest species appear to produce anti-bat sound.

I propose that, prior to the evolution of modern hominids, the moth fauna of caves, particularly the Tineinae fed, as many do now, on bat guano, fungi, and other animal material. Hominids would frequent caves for shelter and rituals (e.g. Dowd, 2008; Pickering et al., 2008) and any keratinophagous moths would undoubtedly exploit the new source of food their clothing and possessions provided. Once, humans began building their own structures these moths would be transferred from caves to these buildings, where they subsequently formed synanthropic populations.

3.5.3 Phylogenetic spread of ATs in Yponomeutoidea

Within the Yponmeutoidea the presence of a hyaline patch between the Cu_{1b} and Cu_2 veins and, by association, sound production, appears to be exclusive to the Yponomeutinae subfamily (Figure 3.5). Secondary loss of the AT appears to have occurred in some taxa following the divergence of *Cedestis* etc. from *Klausius* and the rest of the Yponomeutinae. Some *Cedestis* species and *Zelleria retiniella* possess ATs, whereas other *Zelleria* species and the *Cedestis* species included in the tree do not (Figure 3.5). In the current phylogeny there is no obvious clade for which the lack of ATs is a synapomorphy, meaning it is not possible to infer a single point at which the structure was lost, perhaps suggesting multiple points of secondary loss. A much more detailed phylogenetic analysis of these taxa would be necessary to determine the exact points at which the AT was lost.

Despite possessing a hyaline patch in the same region as yponomeutines, it seems unlikely that *Ochsenheimeria* (Ypsolophidae) species possess the tymbal as it is the only taxon other than the Yponomeutinae possessing a patch. I believe it is coincidentally hyaline due to light coloured or hyaline patterning on the hindwings of these moths. However, microscopic and acoustic investigation would be needed to confirm this.

The driving force behind the evolution of the aeroelastic tymbal is probably the same as sound production and hearing in other moths, predation pressure from bats (Fullard, 1988; Ratcliffe, 2009; Weller et al., 1999). Indeed, a fossil-calibrated lepidopteran

phylogeny suggests the oldest tymbal-possessing Yponomeutinae analysed diverged 51.23 ±17 million years ago (Wahlberg et al., 2013), a similar time to the likely point of echolocation evolution in bats (Jones and Teeling, 2006). However, it is unclear why the aeroelastic tymbal has been lost within the Yponomeutinae. There is no immediately obvious life history trait of *Cedestis*, *Zelleria*, or any other genera within the group lacking the structure that would suggest a biological reason why the trait has disappeared. They are generally nocturnal moths like the rest of the Yponomeutidae (Heppner, 2008), and therefore under predation pressure from bats.

There could be a cost to possessing an aeroleasctic tymbal, which is offset by the anti-bat benefits in genera like *Yponomeuta*, but which is too costly in certain *Cedestis* and *Zelleria* species. This cost could be energetic, sexual, or predatory. Possessing a tymbal may affect the aerodynamic performance of a wing. Wing scales have been shown to enhance aerodynamic performance in Lepidoptera (Slegers et al., 2017), and the aeroelastic tymbal is defined by a lack of scales; however, it is a very small region of the wing, so this would seem unlikely. A predatory cost may arise if the moth is a Batesian mimic of acoustically aposematic moths, none of which are sympatric, meaning any sound production could be a cue to hunting bats rather than a warning. Interestingly though, species of both *Cedestis* and *Zelleria* feed on pine trees (*Pinus* spp.) as larvae (Larsson and Tenow, 1980; Stevens, 1971) the needles of which contain teratogenic alkaloids (Tawara et al., 1993), which could be sequestered by these moths, rendering them toxic. There is no evidence suggesting that these moths sequester secondary metabolites or synthesise their own toxins, but this is merely due to its absence from the current literature. However, this would suggest that the food plants of these moths are not a limiting factor on their toxicity. These genera possess narrower wings than other ypnomeutines and it may simply be that there is not space on the narrowest of *Zelleria* and *Cedestis* wings for a functional AT.

This is merely conjecture, and the dataset (the number of species included in the phylogeny) is not large enough to draw conclusions from with regards to the loss of ATs. The evolutionary history behind the loss of ATs within the Yponomeutinae needs to be investigated further, starting with a detailed molecular phylogeny of the subfamily and analysis of the toxicity and sound-producing capabilities of its member species. However, this analysis does strongly suggest that the AT has a single evolutionary

origin in the superfamily, around the time the Yponomeutinae subfamily began to diverge.

3.5.4 Cave-dwelling microlepidoteran acoustics

The cave-dwelling microlepidoptera *Tinea pellionella*, *Monopis crocicapitella* (both Tineidae), and *Endrosis sarcitrella* (Oecophoridae) all produce ultrasonic clicks powered by their wingbeat. *T. pellionella* and *M. crocicapitella* do so using hyaline patches on their forewings, which most likely function as wingbeat-powered ATs similarly to the hindwing ATs of *Yponomeuta* and their relatives (O'Reilly et al., 2019). *E. sarcitrella* is not very closely related to the Tineidae and does not possess any obvious forewing structures like *T. pellionella* and *M. crocicapitella*. Instead a hyaline patch on the hindwing in the same position as the AT of *Yponomeuta* seems the most likely candidate structure.

Evidence corroborates the idea that the hyaline patches of *T. pellionella*, *M.* crocicapitella, and *E. sarcitrella* function as ATs. Like *Yponomeuta* sounds, the clicks of these moths occur in two bursts every wingbeat, with one burst likely occurring during the upstroke and the other during the downstroke. For both tineid species, ablation of both hyaline patches eliminated sound production, and ablation of one of the two patches did not result in a change in the periodicity of the click bursts, instead halving the total number of clicks per wingbeat. This demonstrates that each tymbal is producing half the total number of clicks per wingbeat, each tymbal contributes to both click bursts, and the body of the moth does not prevent clicks from one wing reaching the opposite side. Although specific ablation of the hyaline patches of *E. sarcitrella* was unsuccessful, the removal of the hindwings eliminated sound production, and there is no other obvious candidate structure on these wings. Additional support comes from the location of the *E. sarcitrella* hyaline patch being identical to that of *Yponomeuta* ATs.

I believe that like other tymbals all three structures produce sound through bimodal buckling, and that the two click bursts each moth produces per full wingbeat are the two stages of its structure buckling and then returning to its resting state. The exact biomechanical mechanism by which the tymbal is actuated was beyond the scope of this study and requires complex modelling, but I propose that, similarly to *Yponomeuta* ATs (O'Reilly et al., 2019), twisting and folding of the wing (likely along flexion lines,

e.g. claval furrow or median flexion line) during flight are important. Strong supporting structures are also probably important, as *Monopis*, *Crypsithyrodes*, and *Crypsithyris* discal cells have thickened veins surrounding their tymbals (Huang et al., 2011; Robinson, 1980).

Structurally, all three tymbals are again similar to *Yponomeuta* ATs; they consist of similarly sized hyaline patches devoid of scales between two veins. However, unlike *Yponomeuta* ATs, they do not possess obvious microtymbals. Microtymbals are striations running the length of a tymbal, each functioning to produce an individual click in sequence following tymbal actuation, resulting in the production of bursts of clicks. Following initial tymbal buckling each microtymbal buckles in sequence producing a train of individual clicks, and then upon the return of the tymbal to its resting state the same process occurs in reverse order, again producing a train of clicks.

The low click number in *T. pellionella* and *E. sarcitrella* click bursts is consistent with a lack of microtymbals; however, the higher click number in *M. crocicapitella* bursts indicates that this species may possess an alternative mechanism. Raised 'bumps' are visible on the ATs of some *Monopis* species, which may be analogues of microtymbals (Figure 3.8), these are visible but less obvious on *M. crocicapitella* (Figure 3.1).

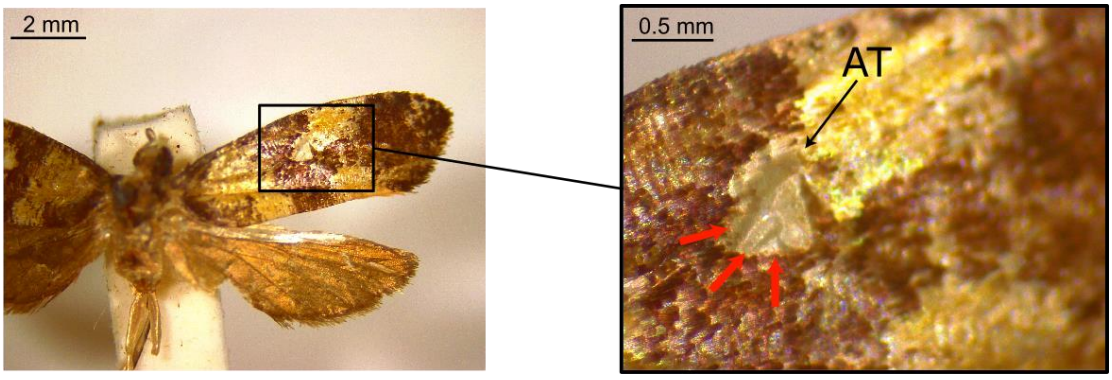


Figure 3.8 *Monopis crateroxantha* AT showing raised 'bumps' (indicated by red arrows) which may act as analogues of the microtymbals of other lepidopteran tymbals. Like microtymbals these bumps may allow for the production of bursts of clicks, as seen in the recordings of *Monopis crocicapitella* sounds.

3.5.4.1 Function of sounds

The acoustic emissions of all three species most likely function as anti-bat sounds. The ultrasonic, broadband nature of the clicks is similar to the known anti-bat sounds of other moths (Barber and Kawahara, 2013; Corcoran et al., 2010; Corcoran and Hristov, 2014; O'Reilly et al., 2019) and they are loud enough to be detected by bats.

The maximum distances at which bats can detect these sounds is lower than *Yponomeuta* clicks (5.9, 4.1, and 3.4m for *M. crocicapitella*, *T. pellionella*, and *E. sarcitrella* respectively and 10.5m for *Y. cagnagella*) (O'Reilly et al., 2019) (see Chapter 2). This is due to increased atmospheric attenuation of the sounds due to much higher peak frequencies, and for *T. pellionella* and *E. sarcitrella* sounds, lower source levels.

T. pellionella and *E. sarcitrella* produce much fewer, lower amplitude clicks per burst (normally one or two, but occasionally more, see Figure 3.6) than *M. crocicapitella*. However, producing fewer clicks does not mean that these sounds are less likely to function as a bat defence. Within the tymbal-possessing Arctiinae, many produce bursts like *M. crocicapitella* but some species, including the sympatric *Arctia caja*, do not possess microtymbals and thus similarly produce one click per tymbal buckling event (Fenton and Roeder, 1974; Surlykke and Miller, 1985).

Additional support for these sounds having an anti-bat function is the lack of any reaction from the moths to ultrasonic stimuli, whether generated artificially or by another individual. Although tympana have been reported in the Tineidae, this is a defining feature of the subfamily Harmacloninae (Davis, 1998), and there is no evidence they are present in the Tineinae. Similarly, there is no evidence in the literature that *E. sarcitrella* possesses hearing capabilities. Therefore, these moths cannot be communicating with conspecifics. Constantly producing ultrasonic clicks that serve no communication purpose, in a closed-off environment such as a cave, shared with an auditory specialist predator seems counterintuitive, unless the sounds act as defence.

How these sounds function defensively is not clear. A lot is dependent on factors not tested here or elsewhere in the literature, for example the toxicity of the moths and how much time they spend on the wing and therefore producing sound. One thing is certain however, the low duty cycles of their sounds prevent them from jamming bat echolocation, as a duty cycle of 20% or more is essential (Conner and Corcoran, 2012; Corcoran et al., 2010). It is also unlikely that bats will be startled by these sounds as they share their roosts with these moths and they undoubtedly hear them regularly and will become habituated to them. This suggests that the sounds function as aposematic signals, as either Batesian (imposter) or Müllerian (true) mimics of acoustically aposematic moths such as the Arctiinae and *Yponomeuta*.

Toxicity in Lepidoptera is derived from sequestering secondary metabolites from food and/or synthesising compounds (Rothschild et al., 1970). As far as I am aware there are no studies describing the toxicity of *E. sarcitrella* or tineid moths, but both faeces and the fungi that grow on it could conceivably provide noxious compounds to sequester. They may also be able to synthesise such compounds.

Bats will learn over time to ignore acoustic Batesian signals (Barber and Conner, 2007), so if these cave-dwelling moths are palatable, they then risk becoming conspicuous targets. Therefore, the persistence and convergent evolution of sound production within this subfamily suggests that these moths are truly aposematic. Toxicity tests as well as palatability tests with bats would be obvious next steps, as would behavioural tests with bats to determine the effectiveness of these sounds as a defence. An interesting possibility is that some naïve juvenile bats first learn to avoid clicking moths from within their roosts.

Alternatively, reducing the exposure of bats to Tineinae acoustic signals could allow for Batesian mimicry to persist. If these moths preferentially avoid flight, and instead crawl atop the guano, they will avoid sound production. This would prevent saturating the bats with a potentially Batesian signal and therefore reducing the effectiveness of sound production as a defence. Additionally, the high peak frequencies of Tineinae clicks may be beneficial in this respect, as it results in reduced detection distances and therefore conspicuousness; only providing the bats with the signal when they are close enough to become a risk.

A second, not necessarily separate, scenario that may allow for the persistence of Batesian mimicry in cave dwelling taxa could arise if the ratio of Müllerian to Batesian mimics bats encounter is so high it is not worth risking attacking clicking targets. If, as the number of recent discoveries appear to show (e.g. Corcoran and Hristov, 2014; Kawahara and Barber, 2015; O'Reilly et al., 2019), the number of sound producing moths is higher than previously thought, then bats will probably be regularly exposed to true aposematic signallers when foraging, reinforcing the effectiveness of acoustic aposematism. Within the roost, palatable cave-dwelling microlepidoptera could then 'piggyback' on the protection afforded by sound production, and reduced conspicuousness and/or flight could maintain the effectiveness of their signals.

Everything considered, based on the similarities of their sounds with those of aposematic moths, their lack of both hearing and intraspecific communication, as well their unusual feeding ecology in close proximity to bats, I conclude that *M. crocicapitella*, *T. pellionella*, their tymbal-possessing relatives (Figure 3.4), and *E. sarcitella* are mimics of acoustically aposematic moths.

3.5.5 Conclusion

Through this project I firstly present the incredible convergent evolution (15 independent evolutionary events) of the newly discovered wing-beat powered sound production mechanism (aeroelastic tymbal) (O'Reilly et al., 2019) within the understudied microlepidoptera. I secondly confirm wing-beat powered sound production in four new species of microlepidoptera in three new families (Tineidae, Oecophoridae, and Depressariidae) using three completely novel wingbeat-powered ATs, and an independently evolved analogue of the *Yponomeuta* AT (O'Reilly et al., 2019). Thirdly, through acoustic characterisation and comparisons with acoustically active moths, I demonstrate that these sounds likely have an acoustic aposematic function as either Batesian or Müllerian mimics of acoustically aposematic moths such as the Arctiinae and Yponomeuta. This project, combined with the studies by Kovalev (2016) and O'Reilly et al.(2019), opens up a new chapter in the bat-moth evolutionary arms race; the anti-bat defences of the microlepidoptera.

Chapter 4: Moths show broadband reductions in ultrasonic echo intensity compared to butterflies: potential acoustic crypsis against bats

Around a third of the acoustic tomography measurements used in this chapter were gathered, under my instruction, by Emily Wood, an MSci student at the University of Bristol whom I co-supervised.

4.1 Abstract

Bats rely on biosonar for hunting success, gleaning information about prey type, size, distance, and velocity from the echoes they receive. Thus, if a prey item can manipulate its echo it can potentially reduce its risk of predation. Lepidoptera are covered in modified setae, scales and hairs. Studies using limited numbers of species show that the setae of nocturnal moths (Lepidoptera most at risk of bat predation) can absorb ultrasound and reduce echo intensity, perhaps providing a form of acoustic camouflage against bats. These moths appear to be more adapted to ultrasound absorption than diurnal Lepidoptera (butterflies and diurnal moths). Here I present the first species-rich phylogenetically spread analysis of the retroreflective echo intensities of butterflies, diurnal moths, and nocturnal moths, supporting the theory that moths are adapted to produce less intense echoes than their diurnal cousins, butterflies. However, diurnal and nocturnal moths do not differ significantly in their echo intensities. This is perhaps attributable to the misnomer of diurnal moths; most traditionally diurnal moths also show partial nocturnal activity and are at some risk of bat predation. Such risk may explain their similar reductions in echo intensity to nocturnal moths. Reductions in echo intensity are widespread in moth taxa, and, therefore, so is potential acoustic camouflage against bats. Moths passively manipulate their echoes to reduce predation risk, whereas diurnal butterflies do not.

4.2 Introduction

During the darkness of night bats are almost completely reliant on biosonar for navigation and prey localisation. Echoes of insects encode vast information to a bat about prey type, size, distance, and velocity (Simmons et al., 1975) and are thus vital to hunting success. So, manipulating echo characteristics could result in a survival advantage for a potential prey item. Velocity and distance information of prey are practically impossible to conceal as they are encoded by hard to manipulate Doppler shift and echo delay respectively (Altringham, 2011). Echo amplitude, however, which encodes prey size should be a simpler characteristic to manipulate by adjusting the amount of sound a target insect reflects.

Echo manipulation by prey in the bat-nocturnal insect arms race is a burgeoning new field of bioacoustic research. With recent discoveries including possible acoustic camouflage by resting moths against rough substrates (Clare and Holderied, 2015), and acoustic wing decoys of certain saturniid moths diverting bat attacks away from their bodies (Barber et al., 2015; Lee and Moss, 2016).

Particular focus, beginning with Zeng et al. (2011), has landed on the ultrasound absorptive potential of lepidopteran setae (Neil et al., 2018; Ntelezos et al., 2017; Shen et al., 2018; Zeng et al., 2011), these appendages generally manifest themselves as scales or hairs and cover the entire wings and bodies of Lepidoptera. These modified setae have several documented functions other than sound absorption. For instance, their pigmentation and structural colouration provide the various visual signals required for their ecology, from predator avoidance to mate attraction (Vukusic and Sambles, 2003). Scales can play roles in pheromone distribution (Clearwater, 1975) and sound production (Barber and Kawahara, 2013; Nakano et al., 2008), and may also help these large-winged insects escape entrapment in spider webs (Nentwig, 1982), regulate their temperatures (Church, 1960; Kingsolver, 1983), and improve aerodynamics (Slegers et al., 2017). Nevertheless, evidence suggests that the wing scales of nocturnal moths absorb more ultrasound within the frequency range of bat calls than both butterflies (Zeng et al., 2011) and diurnal moths (Ntelezos et al., 2017), and that the thorax fur of deaf nocturnal moths absorbs more ultrasound than that of butterflies (Neil et al., 2018 and in press). Neil et al. (2018 and in press) controlled for the effect of body size by using both similarly sized butteflies and moths, but more importantly by calculating

differences within species and comparing the differences between species/lepidoptera type (i.e. butterflies and moths).

Both the studies of Zeng et al. (2011) and Ntelezos et al. (2017) used microreverberation chambers to measure and compare the absorption factors of the wings of nocturnal moths with butterflies and diurnal moths respectively. Zeng et al. (2011) measured the absorption factor of the wings of two moth and two butterfly species with and without scales. They found that the presence of scales on moth wings significantly increased the wing's absorption factor, but scales had no significant effect on butterfly wings. Additionally, removal of scales reduced the moth wings' absorption factors to butterfly wing levels. The absorptive power of moth scales was attributed to the arrangement and ultrastructure of these appendages; butterfly scales are uniform in arrangement and structure, whereas moth scales are arranged in a more haphazard manner and contain perforations absent in their butterfly counterparts (Zeng et al., 2011). Moth scales resemble microperforated panel sound absorbers backed by air space and their arrangement connects these air pockets, perhaps permitting sound to propagate between them allowing sound energy to be dispersed (Zeng et al., 2011).

On a more detailed mechanistic level using laser Doppler vibrometry, Shen et al. (2018) measured the vibrational properties of a saturniid wing scale at frequencies relevant to bat echolocation and showed that three of its resonance peaks were within the frequency range used by bats (27.6, 90.8, and 152.3 kHz). Thus, they suggest that moth scales may achieve their acoustic absorbance as resonant sound absorbers. They also numerically modelled the scale and calculated potential absorption coefficients, which match the acoustic data in the literature.

Ntelezos et al.'s (2017) results corroborate those of Zeng et al. (2011) as absorption factors in Lepidoptera under greater pressure from bat predation were higher (nocturnal vs. diurnal moths). They also found intersex differences in conspecifics, suggesting that sexual dimorphism in flight behaviour may have led to differences in absorption factor within species. For example, males of the saturniid *Samia cynthia ricini*, like other saturniid species, are active during the day searching for sedentary, pheromone-producing females. Post mating, the females take flight at night to find foodplants for oviposition. Thus, female *S. cynthia ricini* are likely to be under greater predation pressure from bats than males are, and Ntelezos et al.'s results show that females of this

species have significantly higher absorption factors for their wings. Female saturniids are generally larger than males and, therefore, may also represent more conspicuous targets in species where both sexes are nocturnal; thus, female-biased absorption may still be present in these species in order to compensate for the louder echoes of females.

Whilst the microreverberation chamber experiments from the literature find significant differences between groups with varying levels of bat predation, the differences they find are spectrally limited. For example, Zeng et al. (2011) show a peak in absorption factor between around 35 and 55 kHz, with no apparent differences at lower frequencies and differences above 55 kHz being much reduced. Ntelezos et al. (2017), show that significant differences between diurnal and nocturnal moths occur at the lower frequencies and cease above 40 kHz. Bat species vary in their echolocation frequencies so it would be expected that a nocturnal moth would possess broadband absorptive properties in order to cover all potential predators and not be spectrally limited. Additionally, the amplitude differences estimated from Zeng et al. (2011) are small, around 2dB. This measurement technique has shown that diel activity appears to influence the absorption factor of lepidopteran wings; however, it does clearly have limitations. The differences measured using this technique are useful but are not ecologically relevant. Measurements of wing fragments and subsequent estimations of amplitude differences are no substitute for retroreflection measurements of whole insects in terms of ecological relevance.

4.2.1 Lepidoptera and bats

Lepidoptera offer an excellent study system for prey echo manipulation for two reasons. Firstly, they possess wing scales and body hair, which offer the potential to act as sound absorbers, and secondly, the order is under a spectrum of bat predation pressure due to varied diel activity of its taxa, providing natural experimental test groups.

Lepidoptera often constitute a large proportion of insectivorous bat diets (e.g. Clare et al., 2009; Dodd et al., 2012; Dodd and Lacki, 2007; Goerlitz et al., 2010), leading to many defensive adaptations in these insects (see Conner and Corcoran, 2012); thus, the bat-moth evolutionary arms race is a textbook example of predator-prey co-evolution. However, lepidopteran taxa vary in their diel activity, and thus exposure to and predation pressure from echolocating bats. They range from strictly diurnal butterflies, to strictly nocturnal moths, with some traditionally diurnal moths actually showing both

diurnal and nocturnal activity (Fullard and Napoleone, 2001; Kawahara et al., 2017). Such differences in predation pressure have led to different levels of defence against bats. This is evident in defences such as anti-bat hearing, which is extremely widespread and convergently evolved in nocturnal moths (see Conner and Corcoran, 2012; Ratcliffe, 2009), but is only found in crepuscular and nocturnal butterflies (extremely uncommon diel activity for butterflies) (Lucas et al., 2014; Yack et al., 2007), with other hearing structures in butterflies used for communication (e.g. Yack et al., 2000). Bats exert such an influence on moth hearing that the sensitivity of their ears are tuned to the specific frequencies of sympatric bat calls (ter Hofstede et al., 2013). Additionally, species of tympanate (acoustically sensitive) moth families endemic to bat-free environments are significantly deafer than immigrant species; relaxed selection due to a lack of bat predation has resulted in secondary loss of hearing (Fullard, 1994; Fullard et al., 2004). Indeed, the initial studies on ultrasound absorbance in Lepidoptera appear to show similar trends to anti-bat hearing in this respect (Ntelezos et al., 2017; Zeng et al., 2011).

4.2.2 Aims

All studies of potential acoustic camouflage in moths to date have focussed on key example species, but none have investigated the spread of this phenomenon across taxa. In addition, whilst microreverberation chamber tests provide a comparison of absorptive powers at ultrasonic frequencies, techniques which measure retroreflection, such as acoustic tomography (see Balleri et al., 2010; Clare and Holderied, 2015), provide a more relevant measure of target echo with respect to bat echolocation. Therefore, the principle aim of this project was to use acoustic tomography to measure and compare the spectral target strength (echo intensities) of a range of macrolepidopteran (Lepidoptera with a wingspan greater than 20mm) taxa under varying levels of predation pressure from bats. Three groups of Lepidoptera were tested: butterflies, diurnal moths, and nocturnal moths. Due to their varying levels of diurnality and therefore predation pressure from bats, I predicted that the echo intensities over all relevant frequencies would be highest in butterflies, lowest in nocturnal moths, and of intermediate levels in diurnal moths (they are not strictly diurnal and generally show some nocturnality). A secondary aim was to compare other predictors of target strength, the ability of species to hear bats and the ‘furriness’ of their bodies. As eared species can rely on their anti-bat hearing as a defence, I predicted that their echo intensities

would be higher than non-eared species which must rely on other methods of anti-bat defence, such as acoustic camouflage. I also predicted that furrier species would produce lower echo intensities as their fur acts to absorb incident sound rather than reflecting it, and if their bodies are adapted to reduce echo intensity, their wings would show similar reductions.

4.3 Methods

4.3.1 Specimen selection

Whole, dried and spread Lepidoptera specimens were used for all data collection, data exist showing insignificant differences between dead and live lepidopteran specimens in acoustic tomography measurements (Neil et al., unpublished), thus the use of dead specimens for this project is justified. Specimens were either borrowed from the collection at the Bristol Museum and Art Gallery, or reared, killed, and spread by me or other members of the BASELab at the University of Bristol. Museum specimens were only chosen if they had been spread to expose all four of their wings, the angle of their right and left wings was close to 180° with respect to each other, i.e. flat, and they were in at least near perfect condition. The same conditions were applied to BASELab specimens but as our group spread them, we were able to control this.

Lepidoptera were divided into three groups for specimen selection, butterflies, diurnal moths, and nocturnal moths. Based on availability the final sample numbers were as follows: butterflies - five families (Nymphalidae 9 species, Pieridae 9, Hesperiidae 9, Papilionidae 9, and Lycaenidae 9), diurnal moths - ten families (Erebidae 10, Uraniidae 4, Sphingidae 3, Lasiocampidae 1, Zygaenidae 4, Notodontidae 2, Endromidae 1, Geometridae 10, Drepanidae 2, and Saturniidae 4), and nocturnal moths - seven families (Saturniidae 7, Sphingidae 3, Lasiocampidae 6, Noctuidae 8, Geometridae 15, Erebidae 3, and Notodontidae 6)

4.3.2 Echo measurements

Echo measurements in the form of spectral target strength (a measure of echo intensity) were made using acoustic tomography (see Chapter 2 section 2.3.7 for details). Specimens were measured from left to right wing at angles between 10 and 170° in 0.5° steps in the vertical plane.

Echo measurements were split between body and wing using a custom MATLAB script (copyright Marc Holderied). Wing measurements are always of the left wing of the insect, body measurements include the entire body, i.e. head, thorax and abdomen.

The measurements were split morphologically as wings and bodies represent very different acoustic reflectors with different potential absorption mechanisms. Wings are large, flat reflectors covered in flat tile-like scales, whereas, bodies are more cylindrical reflectors often covered in hair-like setae.

4.3.3 Statistical analyses

The maximum (strongest) target strength (i.e. the target strength when the moth was normal to the incident sound) was used. Due to the large size of the data set, analyses were not performed on the entire frequency range measured. Initially data were extracted between 20 and 100 kHz as this includes the most relevant frequencies used by the majority of echolocating bats (Yang, 2010). Then the data were divided into sixteen 5 kHz bands by taking the mean value of all constituent frequencies for each band.

As echo strength is directly related to target size, linear regressions were run on the target strength of both left wing and body against the log10 of their corresponding 2-D areas measured from photographs of the specimens using ImageJ (version 1.52p) for each of the 16 frequency bands. Analyses were run on three data sets, the entire data (i.e. all Lepidoptera), just moths, and just nocturnal moths. As a result, linear regressions were run on the target strengths and log10 body and wing area separately for each data set. All statistical analyses were subsequently run on the residuals of these regressions.

For both left wing and body data, comparisons between butterflies, diurnal moths, and nocturnal moths were performed using a two-tailed Kruskal-Wallis one-way analysis of variance, and a Dunn's post-hoc test with Bonferroni correction for each of the 16 frequency bands. All other comparisons were performed using two-tailed Mann-Whitney U tests.

4.4 Results

4.4.1 Morphological compensation

Linear regressions showed significant effects of both log10 wing and body area against their respective target strengths for all tested frequencies and for all three data sets (see Appendices 5-10 for regression equations and R^2 values). As a result, all subsequent analyses were performed on the residuals of these regressions.

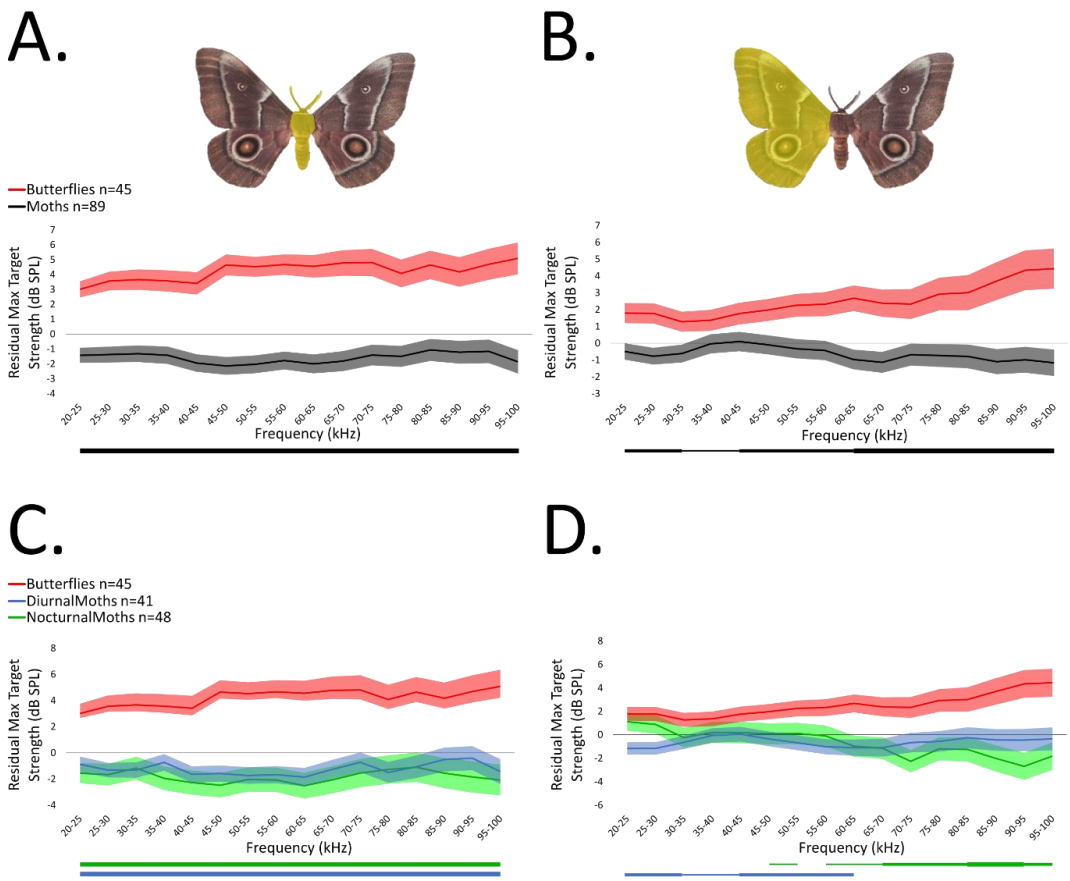


Figure 4.1 Spectral target strength of butterflies, diurnal moths and nocturnal moths All panels show residual maximum target strength (dB SPL) at sixteen 5 kHz frequency bins between 20 and 100 kHz. Target strength is divided by body part with panels A and C representing just body echoes and panels B and D just left wing echoes. Residual values are calculated from linear regressions run on the maximum target strength and log10 of the body or wing area. Panels A and B show data comparing butterflies (red) and moths (black), with panels C and D comparing data from butterflies (red), diurnal moths (blue), and nocturnal moths (green). Solid lines represent median values and shaded areas the standard error. Solid data lines below the data show significance values across the frequency bins and line thickness represents the level of significance. There are three thicknesses, thin ($p=0.01$-0.05), medium ($p=0.001$-0.01), thick ($p<0.001$). For plots C and D, the colour of the significance lines indicates the comparison, with blue lines showing comparisons between butterflies and diurnal moths, and green showing butterflies and nocturnal moths. Significance values were obtained from Mann-Whitney U tests for plots A and B, and Kruskal-Wallis tests followed by Dunn's post-hoc tests with Bonferroni correction for C and D.

4.4.2 Spectral target strength

4.4.2.1 Butterflies and moths

The residual (size compensated) maximum target strength of both the bodies and wings of butterflies is higher than those of moths over all frequencies between 20 and 100 kHz (Figure 4.1a and b). When the moth data are divided by diel activity, differences between butterflies and both diurnal and nocturnal moths in terms of body target strength are maintained over all frequencies (Figure 4.1c); however, the differences in wing target strength differ spectrally between the two groups (Figure 4.1d). Significant differences compared to butterflies are exclusive to diurnal moths at lower frequencies and nocturnal moths at the very highest, with both groups showing significant differences at the mid-range (Figure 4.1d). There were no significant differences in target strength between the two groups of moths across all frequencies for either the body or the wings (Figure 4.1c and d).

4.4.2.2 Other predictors of target strength

Predictors other than Lepidoptera type (butterflies vs. moths) were also analysed, with comparisons made on three data sets; all Lepidoptera (the entire data set), just moths, and just nocturnal moths. Comparisons were made between eared and non-eared as well as 'furry'-bodied and non-furry-bodied for all three datasets, comparisons were also made between day- and night-flying species for the first two datasets.

Comparisons of eared and non-eared Lepidoptera indicate that non-eared species produce significantly higher target strengths than eared species across the entire frequency range for both their bodies and wings (Figure 4.2a and b). The bodies of 'furry' Lepidoptera produce significantly lower target strengths than non-furry species across the entire spectrum (Figure 4.2c), whereas such differences are not mirrored in the wing target strength, but significant differences do exist at the higher frequencies (Figure 4.2d). When comparing day- and night-flying Lepidoptera, the body data shows significantly higher target strengths in day-flying species across the entire spectrum (Figure 4.2e), but such differences are not as strong and only present and significant at the higher frequencies for the wing (Figure 4.2f).

Comparisons of eared and non-eared moths revealed significant differences in body target strength at higher frequencies (Figure 4.3a) but no differences in wing target strength, although there was a similar but statistically insignificant difference (Figure

4.3b). Comparisons of furry- and non-furry-bodied moths revealed no significant differences across all frequencies, but body target strength was generally lower in furry-bodied moths for all frequencies, and wing target strength was lower in non-furry-bodied moths (Figure 4.3c and d). Agreeing with the Kruskal-Wallis and Dunn's post-hoc test results shown in Figure 4.3c and d, the Mann-Whitney U test results comparing day- and night-flying moths show no significant differences in body or wing target strength (Figure 4.3e and f).

In terms of just nocturnal moths, comparisons of eared and non-eared species showed significantly higher target strengths in the bodies of non-eared moths at 80-95 kHz (Figure 4.4a), and no significant differences in wing target strength, although there are apparent statistically insignificant differences at the higher frequencies (60-100 kHz) showing non-eared species producing higher target strengths (Figure 4.4b). There are also no statistically significant differences between furry- and non-furry-bodied nocturnal moths in either body or wing target strength (Figure 4.4c and d). However, non-furry-bodied species show lower target strengths from around 60 kHz onwards for their bodies, and whilst furry-bodied species maintain a fairly constant spectral target strength non-furry species fluctuate across the spectrum (Figure 4.4d).

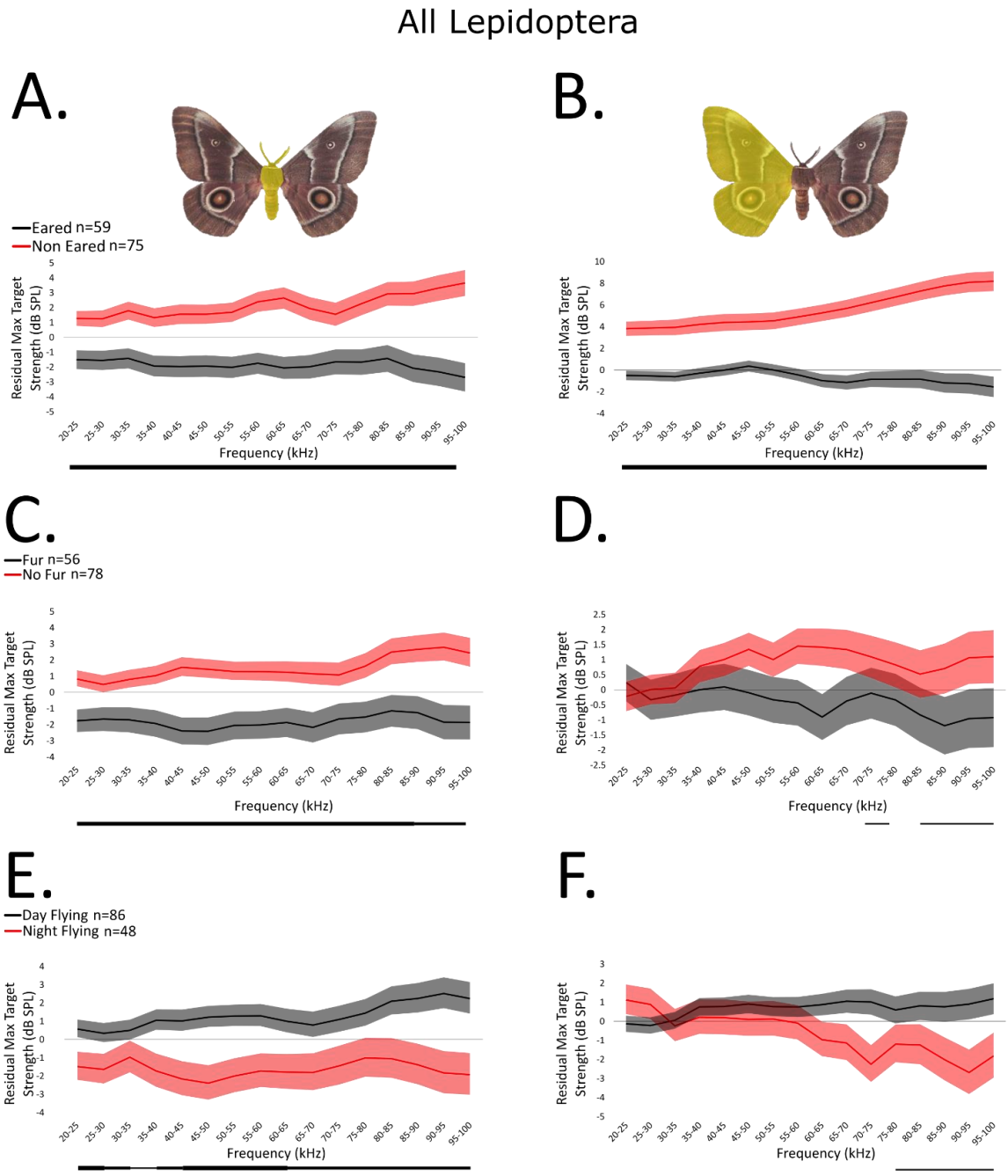


Figure 4.2 Comparisons of three predictors of spectral target across all Lepidoptera All panels show residual maximum target strength (dB SPL) at sixteen 5 kHz frequency bins between 20 and 100 kHz. Target strength is divided by body part with panels A, C, and E representing just body echoes and panels B, D, and F just left wing echoes. Residual values are calculated from linear regressions run on the maximum target strength and log10 of the body or wing area. Panels A and B show data comparing eared (red) and non-eared (black) Lepidoptera, C and D show comparisons of 'furry'-bodied (black) and non-furry-bodied (red) Lepidoptera, and E and F show comparisons of day- and nigh-flying Lepidoptera. Solid data lines represent median values and shaded areas the standard error. Solid lines below the data show significance values across the frequency bins and line thickness represents the level of significance. There are three thicknesses, thin ($p=0.01$-0.05), medium ($p=0.001$-0.01), thick ($p<0.001$). Significance values were obtained from Mann-Whitney U tests.

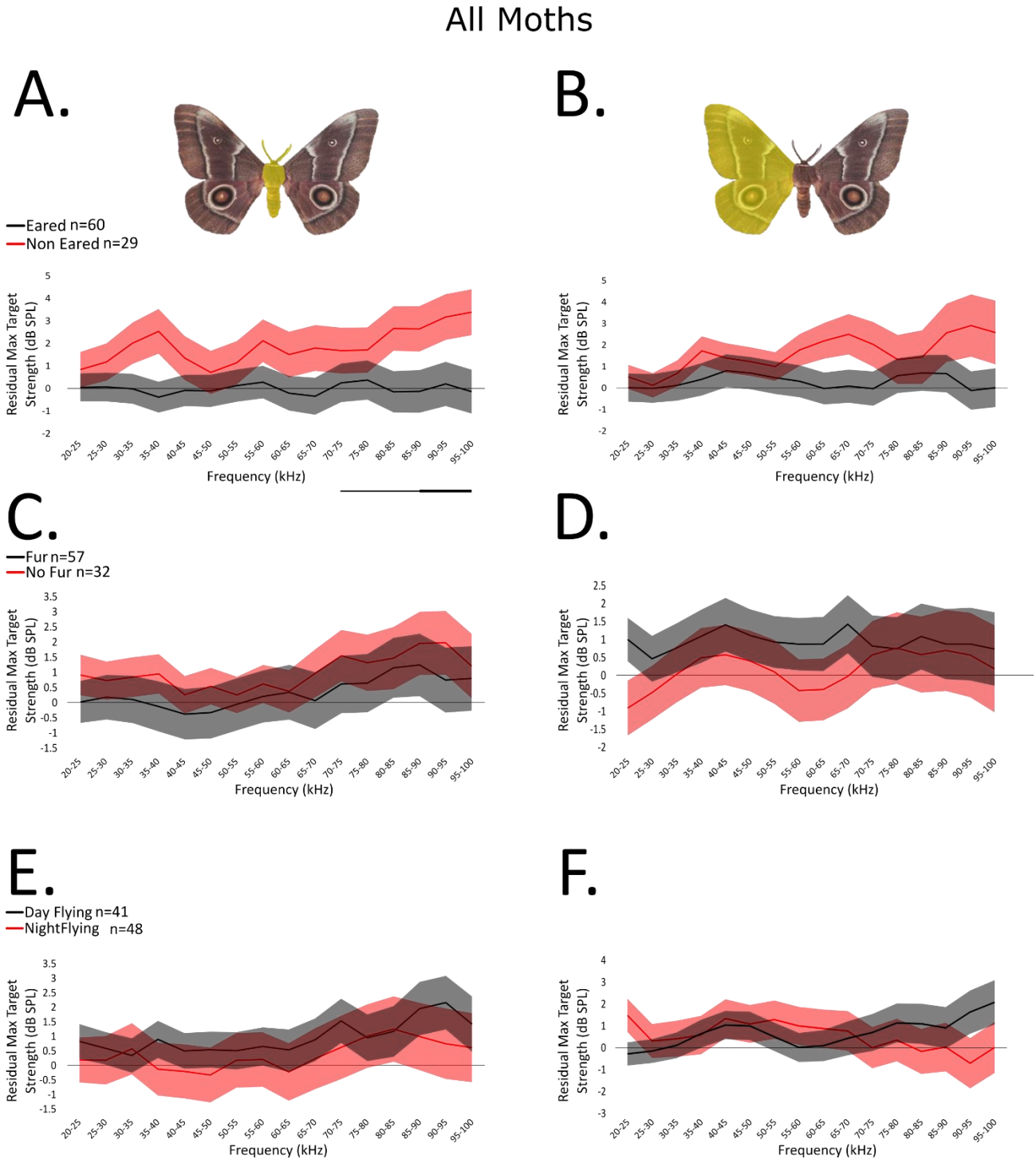


Figure 4.3 Comparisons of three predictors of spectral target across just moths All panels show residual maximum target strength (dB SPL) at sixteen 5 kHz frequency bins between 20 and 100 kHz. Target strength is divided by body part with panels A, C, and E representing just body echoes and panels B, D, and F just left wing echoes. Residual values are calculated from linear regressions run on the maximum target strength and log10 of the body or wing area. Panels A and B show data comparing eared (red) and non-eared (black) moths, C and D show comparisons of 'furry'-bodied (black) and non-furry-bodied (red) moths, and E and F show comparisons of day- and nigh-flying moths. Solid data lines represent median values and shaded areas the standard error. Solid lines below the data show significance values across the frequency bins and line thickness represents the level of significance. There are three possible thicknesses, thin (p=0.01-0.05), medium (p=0.001-0.01), thick (p<0.001), note here only thin and medium are required. Significance values were obtained from Mann-Whitney U tests.

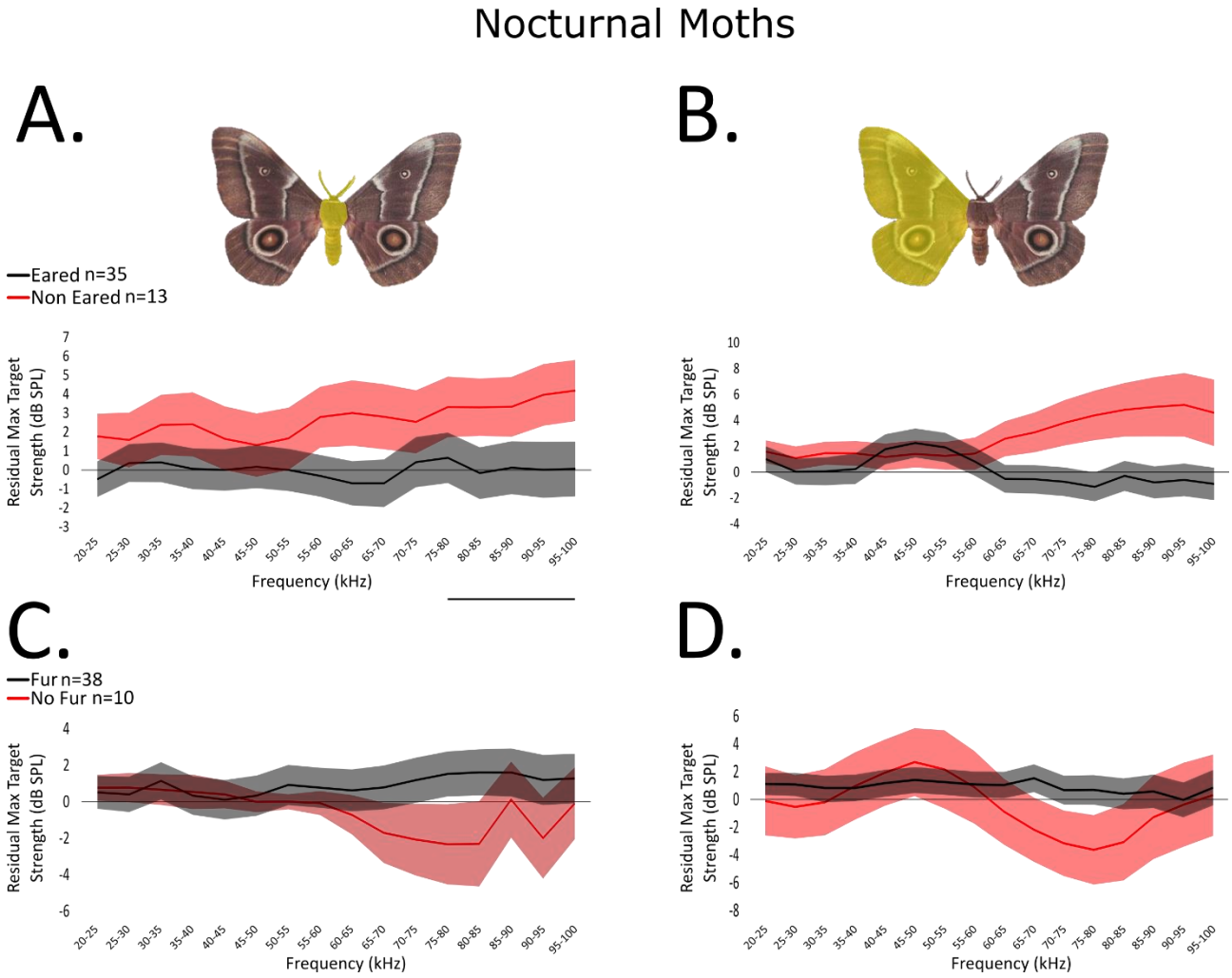


Figure 4.4 Comparisons of two predictors of spectral target across just nocturnal moths All panels show residual maximum target strength (dB SPL) at sixteen 5 kHz frequency bins between 20 and 100 kHz. Target strength is divided by body part with panels A and C representing just body echoes and panels B and D just left wing echoes. Residual values are calculated from linear regressions run on the maximum target strength and log10 of the body or wing area. Panels A and B show data comparing eared (red) and non-eared (black) nocturnal moths, with C and D showing comparisons of 'furry'-bodied (black) and non-furry-bodied (red) nocturnal moths. Solid data lines represent median values and shaded areas the standard error. Solid lines below the data show significance values across the frequency bins and line thickness represents the level of significance. There are three possible thicknesses, thin (p=0.01-0.05), medium (p=0.001-0.01), thick (p<0.001), note here only thin is required. Significance values were obtained from Mann-Whitney U tests.

4.5 Discussion

4.5.1 Butterflies, diurnal moths, and nocturnal moths

When comparing my results to those in the literature it is important to understand that the differences in measurement technique limit the possible comparisons. The two main published studies on this topic used microreverberation chambers to measure the absorption factors of wing fragments (Ntelezos et al., 2017; Zeng et al., 2011), whereas I used acoustic tomography to take retroreflection measurements of whole insects. Therefore, the only true comparisons that can be made are the general trends of echo reduction, i.e. whether nocturnal moths show echo reduction compared to butterflies and diurnal moths, and spectral differences of wings (they did not measure bodies).

My data support the results of Zeng et al. (2011) in that moths produce weaker echoes than butterflies. Unlike their results, where differences were limited to frequencies above around 30 kHz and dramatically peaked between 40 and 50 kHz, I find broadband (20-100 kHz) differences between moth and butterfly wings, I also find the same results for the bodies. My data do not, however, concur with the findings of Ntelezos et al. (2017) as I do not find significant differences between moths based on their diel activity for either body or wing. Although my results do not statistically corroborate their findings comparing diurnal and nocturnal moths, they may suggest a similar trend. The differences between diurnal moths and butterflies are lower than those of nocturnal moths and butterflies at all frequencies for their bodies (3.9-6.5 dB and 4.6-7.2 dB respectively) and from 65-100 kHz for their wings (3.0-4.8 dB and 3.5-7.0 dB respectively). However, spectrally these do not match with the literature; it was at the lower frequencies (20-40 kHz) that Ntelezos et al. (2017) found differences between diurnal and nocturnal moth wings.

As predicted, butterflies produce the strongest echoes of the three lepidopteran groups for both their bodies and wings and this can be attributed to their lack of predation pressure from echolocating bats due to their mostly, and in many species strictly, diurnal activity (Fullard and Napoleone, 2001). These Lepidoptera are therefore unlikely to possess any adaptation to reduce their echoic signature. On the other hand, as predicted, nocturnal moths produced the weakest echoes in terms of their bodies. These Lepidoptera are under the greatest predation pressure from echolocating bats and many of these taxa have evolved defences against bats such as hearing and sound production. Therefore, I believe that the lower amplitude echoes recorded from these

insects are indicative of them having evolved to be less conspicuous to the biosonar of bats.

The fact that diurnal moths are not significantly different to nocturnal moths in terms of either body or wing target strength was not predicted as previous results suggested otherwise (Ntelezos et al., 2017). There are, however, two immediately obvious potential explanations. Firstly, as predicted, the diurnal moths produce stronger (although not significantly) echoes in terms of their bodies over the entire spectrum, and their wings at higher frequencies. Perhaps with a slightly different experimental design these differences may become more pronounced. Diurnal moths were the most difficult of the three lepidopteran groups to source. As a result, some specimens were taken from clades containing mostly nocturnal species, where the diurnal moths were an anomaly. Had I been able to only use specimens from families where diurnal behaviour was a synapomorphy (a trait shared by species of a single clade as well as their most common ancestor), I may have seen results that corroborated Ntelezos et al. (2017) due to increased diurnal specialisation. However, Ntelezos et al. (2017) found significant differences within species, so perhaps this relatedness explanation may not hold.

A second explanation for these results relates to the ecology of these insects. Studies show that traditionally diurnal moths in fact show partial nocturnality (Fullard and Napoleone, 2001; Kawahara et al., 2017), and my results may be due to this mixed diel activity. Unless there is a survival cost to reducing echo intensity whilst being active during the day, then even slight predation pressure from bats may be enough to select for this acoustic camouflage. The slightly higher target strengths seen in the diurnal moths compared to nocturnal species could be due to less intense pressure from bats in this group.

Although I do not believe this to be the case for the results I observed, it is possible that in environments such as islands, where avian predators are absent, diurnal moths are under pressure from bats during daylight hours. On these islands without the risk of predation from raptors, bats are predicted to, and in some cases are observed to fly and forage during the day (e.g. Russo et al., 2011). Diurnal moths endemic to these environments may therefore show weaker echoes than their mainland counterparts which are under reduced pressure from bats. This would be important to consider when

selecting samples for a similar experiment to mine, as island moth species and populations may be under unusual bat predation pressures.

4.5.2 Other predictors of target strength

4.5.2.1 Eared vs. non-eared species

Across all three data sets (all Lepidoptera, just moths, and just nocturnal moths) the general trend is that non-eared species produce higher target strengths across the entire spectrum for both bodies and wings. These differences are most obvious and most statistically significant in the data set containing all Lepidoptera (Figure 4.2a and b) and this is probably due to much higher butterfly values skewing the data, as all butterflies were classified as non-eared. The results from the other two data sets were similar to each other with significantly higher target strengths for bodies seen at frequencies between 75 and 100 kHz and 70 and 100 kHz for moths and nocturnal moths respectively. I predicted that non-eared nocturnal moths would produce weaker echoes than their eared counterparts as their lack of hearing would require them to use other strategies of anti-bat defence, such as reduced echoic conspicuousness. However, this appears not to be the case. Deaf moths may simply use defences other than acoustic camouflage to avoid bat predation, for instance, families of deaf moths have been reported to be less abundant during months of peak bat activity (Yack, 1988). This plus other potentially undiscovered defences may prevent the need for such strong acoustic camouflage as their eared relatives. Additionally, eared moths are clearly under significant predation pressure from bats and have evolved audition as a defence; thus, these moths may in fact be under greater pressure than deaf moths (if they avoid bat activity for example), so reducing their conspicuousness should perhaps not be unexpected as another string to their defensive bow.

4.5.2.2 Furry-bodied vs. non-furry-bodied

As predicted, for the data set containing all Lepidoptera furry-bodied species produce significantly lower target strengths than non-furry bodied for body data and this same trend is also seen in the wings of these insects, though it is only statistically significant at higher frequencies. Although ‘furriness’ should not directly affect the target strength of the wings, it may predict it. If furry-bodied Lepidoptera show reduced body target strength, it stands to reason that their wings should show similar reductions, even if such reductions are achieved through a different method, i.e. scales not fur. However,

these data may again be skewed by the presence of butterflies, which were all classified as non-furry-bodied.

Comparisons using just moths showed a similar trend to the full data set, but the removal of the butterfly data also removed statistical significance, suggesting that those high values were indeed skewing the results. Interestingly, the opposite trend was seen in the wing data, showing that furry-bodied moths in fact produced stronger (although not statistically significantly) echoes across the entire spectrum. As stated above, target strength of the wing is not directly affected by the furriness of the body, but it should offer some predictive value if the furriness has an absorptive role. When comparisons were made using just nocturnal moths the results showed very similar target strengths between 20 and 50 kHz with only non-furry-bodied moths reducing in target strength as the frequency increased (Figure 4.4c and d). However, there was a large amount of error at these frequencies and the sample size was almost a quarter of the furry-bodied moths, which may offer some explanation why the differences were not statistically significant.

The results from the moth and nocturnal moth datasets are perhaps also evidence that the absorptive role of body setae is in fact more complicated than originally thought. Furriness of a specimen was judged by the presence or absence of an obvious layer of thick hair-like setae, but it may be that the more scale-like structures on non-furry-bodied moths have a similar functionality and furriness could be one of several methods of reducing body target strength, or the thickness of fur may also be linked to its other potential functions suggested in the literature such as thermoregulation (e.g. Church, 1960).

I find it most likely that for the nocturnal moth dataset the unbalanced sample sizes have resulted in the much larger error seen in non-furry-bodied moths as well as the unusual values seen at the higher frequencies for the body. I also believe that a combination of this smaller sample size plus the lack of a direct effect of furriness on the wing target strength probably accounts for the undulating spectral target strength values seen in non-furry-bodied moths. To gain a true idea of whether furriness has an effect on target strength, I would suggest further tests using a similar method with balanced sample sizes for furry and non-furry-bodied nocturnal moths. Unfortunately,

as this was not the primary aim of this project, the sample sizes were not balanced for this test.

4.5.2.3 Diel activity

The combination of butterflies and diurnal moths in a comparison with nocturnal moths shows that diel activity has a significant effect on target strength with day-flying Lepidoptera producing stronger echoes at all frequencies for bodies and 80 to 100 kHz for wings. However, these data are again skewed by the inclusion of the much higher butterfly target strengths. Indeed, when the diel activity of moths is compared there are no significant differences, although the body target strengths of nocturnal moths are generally weaker than those of diurnal moths. However, the same is not true for wings, which have stronger target strengths in nocturnal moths at the lowest and mid-range frequencies, and diurnal moths at the higher frequencies. These data have been already been discussed in an ecological context above.

4.5.3 Methods of reducing echo intensity

My data suggest that both the wings and bodies of moths have properties that reduce their potential echo intensity over a large bandwidth of ultrasonic frequencies. Echo intensity could be reduced through absorption, transmission, diffusion or a combination of these. Here I did not test how Lepidoptera may achieve reduced echo intensity but using my results and evidence from the literature I can propose some ideas.

As far as I am aware no study has tested the potential role of transmission; however, results using microreverberation chambers from the literature measured absorption factors and thus showed that absorption at least plays some role in reducing echo intensity in Lepidoptera (Ntelezos et al., 2017; Zeng et al., 2011). So I will focus on absorption from here on. These studies also suggest that wing scales are responsible for this absorption and Neil et al. (2018) found that moth fur appears to reduce thorax echo intensity; thus, I will be focussing on these appendages as the mechanisms by which absorption can occur.

In material science, sound absorption can be achieved through porous absorbers or resonant devices. Both types of absorber offer different spectral limitations. A porous absorber is spectrally limited by its depth in relation to the sound wavelengths and can be poorer at lower frequencies if the depth is insufficient but can be more broadband at higher frequencies. Whereas, a resonant device relies on its natural resonance

frequencies and is thus inherently narrowband in its absorption. Thus, to traditionally create broadband absorption both porous absorbers and resonant devices should be used in tandem (Cox and D'Antonio, 2017), but this may not be necessary in the case of lepidopteran scales and hair.

4.5.3.1 How lepidopteran wing scales may achieve broadband sound absorption

Zeng et al. (2011) suggested that the absorption they observed was the achieved by wing scales acting as both a porous absorber and a perforated panel absorber (a type of resonant device consisting of multiple Helmholtz resonators). However, they and Ntelezos et al. (2017) did not find broadband absorption, which you may expect from such a combination, and which my results suggest.

It is possible that scales may achieve broadband absorption solely as resonant devices. This could be achieved in two ways. Firstly, the resonant device may have multiple resonance peaks at different frequencies, which cover a larger bandwidth when combined, and secondly, resonant devices could act with other resonant devices tuned to different resonance frequencies to form a metamaterial. Similarly to one device having multiple resonance peaks, if multiple resonant devices have different peaks, then together they can provide broadband resonance and absorption.

Interestingly, there is evidence to suggest that wing scales can achieve this feat through one or both of these mechanisms. Shen et al. (2018) showed that one saturniid wing scale has three resonance peaks within the frequency range used by echolocating bats, and when absorption coefficients were calculated from numerical models of the scale, they matched the acoustic results of tests from the literature. This is direct evidence to support multiple resonance peaks for single devices. However, thousands of overlapping scales are present on the wings of moths, and they vary greatly morphologically (Neil et al., in prep. a). Such variation is highly likely to result in different resonance peaks. Thus, a combination of thousands of constituent devices each with different and multiple resonance peaks could easily provide broadband sound absorption as a natural metamaterial.

4.5.3.2 How lepidopteran body 'fur' may achieve broadband sound absorption

Neil et al. (2018) found broadband reductions in echo intensity when thoracic setae are present on moths but not butterflies. When all Lepidoptera are analysed, I find that furriness results in reduced echo intensity; but differences become insignificant when

the analysis excludes butterflies. This suggests that the setae present on butterflies lack the sound absorbing properties of the fur- and scale-like setae of moths. Interestingly, this would mean that even the scale-like body setae of some non-furry-bodied moths can reduce echo intensity. I would suggest that they achieve this through the same proposed methods outlined for wing scales, and that any difference between non-furry-bodied moths and butterflies is due to similar differences in scales to those on the wings.

Fur on the other hand resembles porous sound absorbers such as fibre glass. Theoretically such an absorber should be able to achieve the broadband reductions in echo intensity seen in my results if its depth is sufficient to absorb the longest wavelength (lowest frequency) sounds. However, Neil et al. (in prep. a) modelled the fur of their two test species as traditional porous absorbers and found that it should not be able to achieve the broadband differences they measured. They also found that some of these setae are not entirely hair-like and are in fact similar to wing scales close to their base, and there are sometimes even scale-like setae present amongst the fur. It is therefore possible that the broadband reductions in echo intensity they and I observed could be due to moth fur acting as both a porous absorber, with air gaps between setae analogues of pores in traditional absorbers, and the scale-like bases and setae acting as resonant devices perhaps covering the lower frequencies.

4.5.4 Acoustic camouflage

It is important to discuss how moths may use reduced echo intensity to aid in their survival. Roeder first tested the effect of moth wing scales on wing echo intensity, finding that their presence reduced echo amplitude by 1-2 dB (Roeder, 1962). However, as these amplitudes were considerably lower than the differences associated with wing movement during flight, he concluded they were insignificant. Nevertheless, reducing overall echo intensity could be beneficial to moths and later research, including this work, suggests that effects may in fact be much stronger than Roeder initially measured.

Reduced echo intensity could theoretically provide defence at two stages of a bat's hunting process, firstly reducing initial detectability (crypsis), and secondly by causing misidentification of the prey item (mimesis). Bats, like all animals capable of audition, possess specific hearing thresholds at all frequencies (Heffner and Heffner, 2007; Neuweiler et al., 1984), which can be represented as an audiogram (Figure 4.5). By reducing echo intensity, the distance over which this echo drops below the hearing

threshold, and the target is detectable by a bat, decreases. Thus, reduced echo intensity can result in acoustic crypsis. Therefore, compared to apparently non-acoustically adapted butterflies, moths appear to show acoustic crypsis against bats.

Mimesis could occur, not through mimicry of an inanimate object such as a leaf (although this could be possible, but not necessarily through reduced echo intensity), but of a less profitable prey item. Echo intensity is directly related to target size (Firzlaff et al., 2007), and it is possible that bats use a combination of echo intensity and delay (a measure of target distance) to determine target size. Therefore, in reducing its echo intensity, a potential prey item could provide a dishonest representation of its profitability. Bats will preferentially attack larger prey items when available (Anthony and Kunz, 1977), thus reducing apparent size and, therefore, profitability may reduce predation risk.

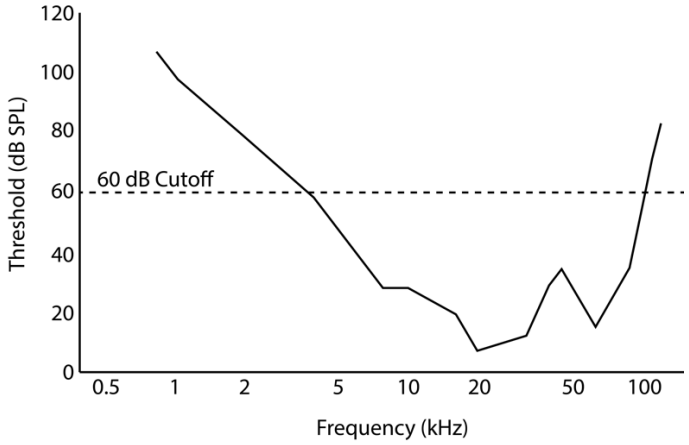


Figure 4.5 Audiogram of big brown bat (*Eptesicus fuscus*) Behavioural audiogram of *E. fuscus* modified from (Koay et al., 1997). The dashed line represents a 60 dB (comparative standard) threshold, where the audiogram crosses this line indicate the upper and lower frequency limits of the bat's hearing.

This theory does not account for spectral indications of prey size. For strong acoustic reflection a target must be larger than the wavelength of the incident sound or the sound will undergo Rayleigh scattering with little to no echo received by the bat (Pye, 1993). Therefore, truly small targets would not be able to reflect lower frequency ultrasound (e.g. 20-30 kHz) and so even a weak intensity echo that contained low frequencies could be interpreted by a bat as a large prey item. However, it is not known whether bats do use this information to determine prey size. Therefore, I cannot conclude whether moths employ acoustic mimesis here

4.5.5 Conclusion

Moths, both diurnal and nocturnal, show reduced echo intensity over the entire frequency spectrum chiefly associated with bat echolocation calls (20-100 kHz). Their bodies and their wings offer two different types of reflectors and these differences are seen in both these morphological regions. Modified setae, scales and hairs (fur), are likely to be responsible for these reduced echo intensities. Scales possibly act as resonant devices fucntioning together as a sound absorbing metamaterial on wings, and in some cases bodies, of moths, and the thick fur of some moths may act as a porous sound absorber in conjunction with scale-like setae to create broadband sound absorption. For the first time, I use a large phylogenetically spread data set to show that moths are significantly less echoically conspicuous than butterflies over the entire frequency spectrum relevant to bat echolocation, but also, contrary to the only previous study, the diel activity of moths does not appear to dictate echo intensity.

Chapter 5: General Discussion

5.1 Novel anti-bat sound production in *Yponomeuta*

Recent research into the defences of moths against echolocating bats has shown that anti-bat sound production is not exclusive to the well-studied, acoustically active Arctiinae, instead it is present in the Geometridae, Sphingidae, and *Yponomeutidae* too (Barber and Kawahara, 2013; Corcoran and Hristov, 2014; Kawahara and Barber, 2015; O'Reilly et al., 2019). Through Chapter 2 I show that *Yponomeuta* moths produce anti-bat ultrasound and that they are Müllerian mimics of the Arctiinae. *Yponomeuta* can advertise their secondary defences (containing noxious compounds such as butenolides) whilst bypassing the need for predator detection. This discovery provides many firsts in terms of the bat-moth evolutionary arms race. It is the first example of anti-bat ultrasound in a deaf moth and in the microlepidoptera (a much-understudied group in this arms race), the first example of constitutive acoustic aposematism, and the first example of the novel sound producing structure, which I am terming the aeroelastic tymbal (AT).

This discovery opens many new avenues of research in both the bat-moth evolutionary arms race, as well as interdisciplinary research. Other than the well-studied pyralid hearing (e.g. Skals and Surlykke, 2000), this is the first strong evidence of advanced acoustic defences in the microlepidoptera. Until now this suborder has largely been overlooked in terms of their anti-bat adaptations; however, I show that this group requires significant investigation in this respect. Due to the pressures from echolocating bats, microlepidoptera, like their larger cousins, have probably evolved multiple acoustic defences against bats.

In terms of interdisciplinary interest, the aeroelastic tymbal represents a natural structure, which allows for aeroelastic sound production through what is likely a buckling mechanism, the implications of which could be great for material science. The actuation mechanism likely comes from the aerodynamic forces of the wing during the wingbeat and the elastic properties of the AT. There are likely to be many technological applications for this, for example, echolocating drones that passively produce their sound through flight, negating the need for a speaker. Additionally, buckling structures in general are of significant interest, particularly in terms of "smart" applications, i.e. materials which change properties depending on conditions (Hu and Burgueño, 2015).

The aeroelastic tymbal may represent yet another example of bioinspiration for engineering.

5.2 Aeroelastic tymbals in the microlepidoptera

I immediately followed up on the discovery of the AT in *Yponomeuta*; predicting that such an elegant solution for acoustic aposematism in deaf moths would be more common. I explored the phylogenetic spread of ATs in the microlepidoptera, finding by visual inspection that they are indeed widespread. The structure appears to have evolved independently in this suborder at least 15 times, and is found in four regions of the wing, three on the forewing and one on the hindwing. I confirmed sound production from all five AT examples I was able to test (including *Yponomeuta*), suggesting that other examples I found through morphological examination but was unable to test acoustically, are likely to also function as ATs.

Interestingly, troglophilic (cave-dwelling) ecology appears to be a common trait associated with AT-possessing taxa. These taxa are keratino- and/or guanophagous and often are part of the food webs based on bat guano in caves. Therefore, they exist alongside their potential predators, putting them at perpetual risk of predation. It is this unusual feeding ecology, which is widespread in the tineid subfamily Tineinae, that I believe is responsible for the multiple independent evolutionary origins of ATs in this group, an unprecedented three cases of convergent evolution in one subfamily. Further evidence to support that their troglophilic ecology is responsible for this convergence comes from *Endrosis sarcitrella* (Oecophoridae), a distantly related species from a different superfamily. *E. sarcitrella* shares a similar troglophilic ecology to the Tineinae and produces similar sounds through an unconfirmed AT.

Another interesting finding from the phylogenetic analysis is the convergence of ermine colouration (black/dark spots on a white/light background) in AT-possessing taxa. This could represent an aposematic colouration, and if so, these moths could have converged on the same acoustic and visual warning signals. Ermine colouration is described in the literature as both aposematic (Marsh and Rothschild, 1974) and not (Menken et al., 1992). I believe the convergence of this colouration in species that are probably acoustically aposematic suggests that it is a visual aposematic signal. Further work characterising these ermine coloured moths acoustically and visually, as well as quantifying their aposematic quality with predators in both sensory modalities, would

provide a textbook example of aposematic mimicry in two sensory modalities possibly against two types of predator. Even a study that just quantifies the survival advantage afforded by the visual and acoustic aposematic signals of one species such as an *Yponomeuta* sp. would provide evidence to support the Perceptual Variability Hypothesis of the evolution of multimodal aposematism (Rowe and Halpin, 2013). The evolution of multimodal aposematism is an unclear area of sensory and evolutionary biology and the Perceptual Variability Hypothesis "still awaits conclusive evidence" (Rowe and Halpin, 2013). This hypothesis postulates that a multimodally aposematic organism's warning signals evolved to warn multiple predators that use different sensory modalities, in the case of AT-possessing moths this would be visual signals for birds, invertebrates, or rodents and acoustic signals for bats. Chapter 2 could therefore pave the way for a study into an important aspect of predator-prey ecology.

Chapter 3 also presents the first detailed molecular phylogeny of the Tineidae. However, this phylogeny was only constructed using one protein; thus, a more robust phylogeny should be made for future studies, ideally using multiple mitochondrial and nuclear proteins. Nevertheless, it is a reference point until such a phylogeny is created.

5.3 Moths are less echoically conspicuous than butterflies

I set out to provide a species-rich, phylogenetically spread analysis of the differences in echo intensity of butterflies, diurnal moths, and nocturnal moths. Previous studies with few species had suggested that nocturnal moths are adapted to be less conspicuous to bat biosonar than butterflies (Neil et al., 2018; Zeng et al., 2011), and diurnal moths (Ntelezos et al., 2017) through reduced echo intensity. I analysed acoustic tomographies of whole insects and divided them into body and wing data finding that the bodies of nocturnal moths were consistently less echoic than butterflies over all frequencies between 20 and 100 kHz (frequencies used in bat echolocation), and their wings were too at higher frequencies in this range (50-100 kHz excluding 55-60). My data did not, however, statistically support that nocturnal moths were less echoic than diurnal moths. In fact, similarly to nocturnal moths, diurnal moths produced significantly weaker echoes than butterflies over all tested frequencies for their bodies, and their wings at lower frequencies. Also, due to the lack of significant differences between nocturnal and diurnal moths for both their bodies and wings over all tested frequencies, I pooled the moth data and found significantly lower echo intensities over the entire spectrum

for both bodies and wings than butterflies. When the data were pooled the results showed very strong support for Zeng *et al.*'s findings that moth wings absorb more ultrasound than butterfly wings However, my data provide a much stronger picture; Zeng *et al.* (2011) only found small differences (estimated to be around 2dB at maximum) and these differences were limited to about 40-50 kHz. Whereas, I show that differences are in fact broadband (20-100 kHz) and much greater in amplitude (4.4-6.9dB SPL for the body and 1.4-5.6 dB SPL for the wings when moth data are pooled).

Previous work has suggested that the fur on moths helps to provide absorptive power (Neil et al., 2018). My results suggest that it may do, but also that the more scale-like setae on moth bodies could provide similar absorption. However, I did not measure absorption here, just size compensated echo intensity and so I cannot draw conclusions on whether this is in fact absorption. Differences in echo intensity could be due to absorption, diffusion, or transmission, and different studies are needed to untangle the exact mechanism. Nevertheless, my results do suggest that scale-like body setae on moths provide similar echo reductions to body fur.

As I was unable to remove the setae on the specimens I tested, I could not confirm that they were providing the echo reduction. However, as previous studies have shown both wing scales and body fur provide reductions (Neil et al., 2018; Zeng et al., 2011), and Shen *et al.* (2018) demonstrated wing scales could provide absorption as resonant devices, I believe they are responsible for the results I saw with this study.

5.4 Future research

5.4.1 Aeroelastic tymbals

My discovery of the widespread, convergently evolved ATs in microlepidoptera presents several avenues for future research. Firstly, the anti-bat functionality should be tested, and its effectiveness quantified, through behavioural tests with bats. Whilst the similarities with known anti-bat sounds such as those of the Artciinae combined with the ecology of these moths strongly suggests an anti-bat function, I have not tested this here. Additionally, as mentioned above, it would be interesting to explore the potential multimodal aposematism of ermine coloured AT-possessing taxa. These species could represent perfect model species for investigating the Perceptual Variability hypothesis of multimodal aposematism (Rowe and Halpin, 2013).

Secondly, the association of ATs with cave-dwelling, guanophagous taxa, particularly the three cases of convergent evolution in one subfamily, implies that cave-dwelling invertebrates are under predation pressure from bats. Many invertebrates, not just Lepidoptera, inhabit cave systems and bat roosts globally (e.g. Humphreys and Eberhard, 2001; Polak, Bedek and Ozimec, 2012; Eberhard *et al.*, 2014); thus, at least the flying taxa should be under similar threat from bats and could have also evolved defences. An investigation into the potential anti-bat defences of cave-dwelling invertebrates would be an interesting study and offers the potential to open completely new chapters in both the bat-invertebrate arms race and the field of cave ecology.

Thirdly, material scientists and engineers are interested in ATs. The structures probably produce their sounds through a buckling mechanism, and although buckling has previously been avoided in smart application design, it is increasingly being exploited (Hu and Burgueño, 2015). I have already collaborated with material scientists at the University of Bristol and a PhD student is currently modelling the *Yponomeuta* AT as their project.

5.4.2 Acoustic camouflage

Obviously, the topic of acoustic camouflage lends itself to technical applications. On a broad level, my research shows that something about moths but not butterflies reduces their echoic presence, probably their modified setae (scales and hairs). In a defence context, these properties could be applied to anti-radar stealth systems to reduce detectability of an object such as a vehicle. In an architectural context, these appendages could be modified to reduce reverberation at frequencies relevant to human hearing, perhaps providing elegant, thin (in the case of moth scales) soundproofing systems. The latter is currently being investigated by members of the BASELab.

In terms of biology, my findings confirm that the moths are generally less echoically conspicuous than butterflies. However, I did not determine whether it was in fact the scales and hairs on these insects that provide this adaptation. Therefore, a detailed investigation into the effect of scale and hair presence on echo intensity should be performed, and indeed the BASElab have performed such investigations (Neil *et al*., in prep a and b).

I found similar reductions in echo intensity in moths with and without furry bodies; thus, a detailed investigation into the effectiveness of different types of body setae as

sound absorbers in moths would be another obvious next step. Additionally, as my data disagree with Ntelezos et al. (2017) in that I do not find significant differences between nocturnal and diurnal moths, it would be pertinent to further study these two groups in detail using acoustic tomography. I would also suggest that such a study used strictly diurnal groups rather than a mixture of strictly diurnal groups and diurnal taxa from predominantly nocturnal groups. In addition, a study on the echoic characteristics of flying moths would be useful to show how echo strength varies dynamically during flight; another step towards realism in perceptual space modelling of bat prey. Thus far, most research has assumed moths benefit from the absorption of ultrasound as it reduces their conspicuousness to echolocating bats in flight. However, this theory only applies when the background is non-reflective, i.e. air. When a moth rests on a surface, its background is reflective; an absorptive moth would be conspicuous to a bat as a less reflective spot on the surface. Perfect acoustic camouflage in moths would be adaptive, providing ultrasound absorbance during flight and reducing this absorbance at rest. Theoretically this could be possible if moths can control the resonant properties of their scales, allowing them to absorb ultrasound in flight, but reflect it at rest. An investigation into whether acoustic camouflage is adaptive in moths would be an important step in fully understanding this anti-bat adaptation. Indeed, a study comparing the echo intensities of moths which vary in the volant activity levels would also be of value. Preliminary studies of adaptive acoustic camouflage have are being performed by members of the BASELab.

There is no direct behavioural evidence that moths are less detectable to bats than butterflies, or that scales or hair have any real-world effect on bat-moth interactions. Therefore, I would also propose behavioural tests with bats to quantify the effectiveness of any acoustic camouflage in moths.

Further to these suggestions, which I believe are of the most immediate importance in this field, a large-scale study of the echo intensities and detectability of whole nocturnal flying insect communities would be interesting. These data could be used with sympatric bat calls and hearing thresholds, to model the expected prey consumption for each bat species. Combining this with prey abundance data and DNA diet analysis from faeces, a comparison could be made with predicted and measured prey consumption. Any discrepancies could then be investigated further. This would be a novel

investigation method for exploring nocturnal community ecology, and possible adaptations to prey detection and capture in bats, or predator avoidance in flying insects.

Additionally, paired comparisons of nocturnal and diurnal flying insect taxa could be performed. A similar study to mine could easily be run using acoustic tomography to compare diurnal and nocturnal taxa from insect orders other than the Lepidoptera, which are under predation pressure from echolocating bats, such as Coleoptera and Diptera. Although there is no direct evidence to suggest that these groups possess acoustic camouflage, it would be worth investigating. Some beetles possess scales on their elytra (wing cases) (Sun and Bhushan, 2012), which could act analogously to moth scales, and some elytra contain pores which could act as Helmholtz resonators allowing the elytra to function as perforated sound absorbers (Cox and D'Antonio, 2017; Sun and Bhushan, 2012). Flies can possess hair like-setae, although there is no evidence to suggest that increased numbers of hairs are associated with nocturnal behaviour. Nevertheless, a preliminary study into this should be straightforward using museum or display specimens.

5.5 Conclusion

Here I provide key findings on passive anti-bat defences in the bat-moth evolutionary arms race. I present the discovery and acoustic characterisation of the first passive anti-bat sound-producing organ, the aeroelastic tymbal. I define ATs as passive despite them being powered by flight as AT sound production is involuntary for the moths. I also show that this structure has likely convergently evolved an incredible 15 times in the understudied microlepidoptera, including three times in one subfamily. These discoveries open up the microlepidoptera as an untapped source of acoustic anti-bat defences, hopefully research interest in these taxa will be increased following this work. From noisy moths I then present data on quiet ones. The first species-rich analysis on the echo intensities of moths and butterflies provides strong evidence that moths are adapted to be less echoically conspicuous to bats over a broadband of relevant ultrasonic frequencies. For the first time evidence is provided across a phylogenetically spread data set, not just a few select species. Acoustic camouflage in moths, therefore, appears to be a widespread phenomenon. Whether loudly or quietly, moths can reduce their chances bat predation through passive means. The bat-moth evolutionary arms race has been the subject of decades of research in many aspects of biology, from

community, behavioural, and sensory ecology to molecular genetics and neurobiology, and yet the research community is still revealing fascinating adaptations on both sides. Undoubtedly, we still have much to uncover about the relationship between moths and their chiropteran adversaries.

References

Agassiz, D.J.L., 2017. Do small ermine moths sing? Possible stridulatory sound production in Yponomeutidae (Lepidoptera). J. Nat. Hist. 1–8. https://doi.org/10.1080/00222933.2017.1324063

Agee, H.R., 1971. Ultrasound produced by wings of adults of Heliothis zea. J. Insect Physiol. 17, 1267–1273. https://doi.org/10.1016/0022-1910(71)90192-2

Ahlen, I., 1997. Fältobservationer av ultraljud hos flygande fjärilar. Entomol. Tidskr. 118.

Alcock, J., Bailey, W.J., 1995. Acoustical Communication and the Mating System of the Australian Whistling Moth Hecatesia Exultans (Noctuidae, Agaristinae). J. Zool. 237, 337–352.

Alcock, J., Gwynne, D.T., Dadour, I.R., 1989. Acoustic signaling, territoriality, and mating in whistling moths, Hecatesia thyridion (Agaristidae). J. Insect Behav. 2, 27–37. https://doi.org/10.1007/BF01053616

Altringham, J.D., 2011. Bats: From Evolution to Conservation. Bats From Evol. to Conserv. 1–352. https://doi.org/10.1093/acprof:osobl/9780199207114.001.0001

Anthony, E.L.P., Kunz, T.H., 1977. Feeding Strategies of the Little Brown Bat , Myotis lucifugus , in Southern New Hampshire. Ecology 58, 775–786.

Bailey, W.J., 1978. Resonant wing systems in the Australian whistling moth Hecatesia (Agarasidae, Lepidoptera). Nature. https://doi.org/10.1038/272444a0

Balleri, A., Griffiths, H.D., Woodbridge, K., Baker, C.J., Holderied, M.W., 2010. Bat-inspired ultrasound tomography in air, in: Radar Conference, 2010 IEEE. pp. 44–47.

Barber, J.R., Chadwell, B.A., Garrett, N., Schmidt-French, B., Conner, W.E., 2009. Naïve bats discriminate arctiid moth warning sounds but generalize their aposematic meaning. J. Exp. Biol. 212, 2141–2148. https://doi.org/10.1242/jeb.029991

Barber, J.R., Conner, W.E., 2007. Acoustic mimicry in a predator-prey interaction. Proc. Natl. Acad. Sci. U. S. A. 104, 9331–9334.

Barber, J.R., Kawahara, A.Y., 2013. Hawkmoths produce anti-bat ultrasound. Biol. Lett. 9, 1–5.

Barber, J.R., Leavell, B.C., Keener, A.L., Breinholt, J.W., Chadwell, B.A., McClure, C.J.W., Hill, G.M., Kawahara, A.Y., 2015. Moth tails divert bat attack: Evolution of acoustic deflection. Proc. Natl. Acad. Sci. 112, 2812–2816. https://doi.org/10.1073/pnas.1421926112

Barr, T.C., Reddell, J.R., 1967. The Arthropod Cave Fauna of the Carlsbad Caverns Region, New Mexico. Southwest. Nat. 12, 253–273.

Bates, D.L., Fenton, M.B., 1990. Aposematism or startle? Predators learn their responses to the defenses of prey. Can. J. Zool. 68, 49–52. https://doi.org/10.1139/z90-009

Bates, H.W., 1862. Contributions to an insect fauna of the Amazon valley (Lepidoptera: Heliconidae). Trans. Linn. Soc. London 23, 495–566.

Bernays, E.A., Oppenheim, S., Chapman, R.F., Kwon, H., Gould, F., 2000. Taste sensitivity of insect herbivores to deterrents is greater in specialists than in generalists: A behavioral test of the hypothesis with two closely related caterpillars. J. Chem. Ecol. 26, 547–563. https://doi.org/10.1023/A:1005430010314

Blest, A.D., Collett, T.S., Pye, J.D., 1963. The generation of ultrasonic signals by a new world arctiid moth. Proc. R. Soc. B Biol. Sci. 158, 196–207. https://doi.org/10.1098/rspb.1963.0042

Bogdanowicz, W., Fenton, M.B., Daleszczyk, K., 1999. The relationships between echolocation calls, morphology and diet in insectivorous bats. J. Zool. 247, 381–393.

Boppré, M., 1990. Lepidoptera and pyrrolizidine alkaloids Exemplification of complexity in chemical ecology. J. Chem. Ecol. 16, 165–185. https://doi.org/10.1007/BF01021277

Brokerhof, A.W., Morton, R., Jonathan Banks, H., 1993. Time-mortality relationships for different species and developmental stages of clothes moths (Lepidoptera: Tineidae) exposed to cold. J. Stored Prod. Res. 29, 277–282.

https://doi.org/10.1016/0022-474X(93)90011-R

Byun, B.K., Shin, S.B., Bae, Y.S., Kim, D.S., Choi, Y.G., 2014. First discovery of a cave-dwelling Tineid moth (Lepidoptera, Tineidae) from East Asia. J. For. Res. 25, 647–651. https://doi.org/10.1007/s11676-014-0503-9

Carter, D.J., 1984. Pest Lepidoptera of Europe with special reference to the British Isles. Dr. W. Junk, Dordrecht, Netherlands.

Centelles Bascuas, R., 2015. Les papillons des grottes Essai d'inventaire français et européen et indications bibliographiques. Spelunca 140, 1–3.

Church, N.S., 1960. Heat loss and the body temperatures of flying insects II. Heat conduction within the body and its loss by radiation and convection. J. Exp. Biol. 37, 186–212.

Clare, E.L., Fraser, E.E., Braid, H.E., Fenton, M.B., Hebert, P.D.N., 2009. Species on the menu of a generalist predator, the eastern red bat (Lasiurus borealis): Using a molecular approach to detect arthropod prey. Mol. Ecol. 18, 2532–2542. https://doi.org/10.1111/j.1365-294X.2009.04184.x

Clare, E.L., Holderied, M.W., 2015. Acoustic shadows help gleaning bats find prey, but may be defeated by prey acoustic camouflage on rough surfaces. Elife 4, 1–14. https://doi.org/10.7554/eLife.07404

Clearwater, J.R., 1975. Structure development and evolution of the male pheromone system in some Noctuidae (Lepidoptera). J. Morphol. 146, 129–175. https://doi.org/10.1002/jmor.1051460107

Cokendolpher, J.C., Polyak, V.J., 2004. Macroscopic invertebrates of Hidden and Hidden Chimney caves, Eddy County, New Mexico. Texas Meml. Museum, Speleol. Monogr. 6, 175–198.

Cokendolpher, J.C., Polyak, V.J., 1996. Biology of the caves at Sinkhole Flat, Eddy County, New Mexico. J. Cave Karst Stud. 58, 181–192.

Conner, W.E., Corcoran, A.J., 2012. Sound strategies: the 65-million-year-old battle between bats and insects. Annu. Rev. Entomol. 57, 21–39. https://doi.org/10.1146/annurev-ento-121510-133537

Corcoran, A.J., Barber, J.R., Conner, W.E., 2009. Tiger moth jams bat biosonar. Science (80-.). 325, 325–328. https://doi.org/10.1126/science.1174096

Corcoran, A.J., Barber, J.R., Hristov, N.I., Conner, W.E., 2011. How do tiger moths jam bat sonar? J. Exp. Biol. 214, 2416–2425.

Corcoran, A.J., Conner, W.E., Barber, J.R., 2010. Anti-bat tiger moth sounds: Form and function. Curr. Zool. 56, 358–369.

Corcoran, A.J., Hristov, N.I., 2014. Convergent evolution of anti-bat sounds. J. Comp. Physiol. A 200, 811–821. https://doi.org/10.1007/s00359-014-0924-0

Cox, T.J., D'Antonio, P., 2017. Acoustic absorbers and diffusers : theory, design and application.

Davis, D.R., 1998. A world classification of the Harmacloninae, a new subfamily of Tineidae (Lepidoptera: Tineoidea). Smithson. Contrib. to Zool. 597, 1–81. https://doi.org/10.5479/si.00810282.597

Dodd, L.E., Chapman, E.G., Harwood, J.D., Lacki, M.J., Rieske, L.K., 2012. Identification of prey of Myotis septentrionalis using DNA-based techniques. J. Mammal. 93, 1119–1128. https://doi.org/10.1644/11-MAMM-A-218.1

Dodd, L.E., Lacki, M.J., 2007. Prey consumed by Corynorhinus townsendii ingens in the Ozark Mountain region. Acta Chiropterologica 9, 451–461. https://doi.org/10.3161/1733-5329(2007)9[451:PCBCTI]2.0.CO;2

Dowd, M.A., 2008. The use of caves for funerary and ritual practices in Neolithic Ireland. Antiquity 82, 305–317. https://doi.org/10.1017/S0003598X00096824

Dowdy, N.J., Conner, W.E., 2019. Characteristics of tiger moth (Erebidae: Arctiinae) anti-bat sounds can be predicted from tymbal morphology. Front. Zool. 16, 1–11. https://doi.org/10.1186/s12983-019-0345-6

Dowdy, N.J., Conner, W.E., 2016. Acoustic Aposematism and Evasive Action in Select Chemically Defended Arctiine (Lepidoptera: Erebidae) Species: Nonchalant or Not? PLoS One 11. https://doi.org/10.1371/journal.pone.0152981

Dudley, R., 2000. The Biomechanics of Insect Flight. Princeton University Press. https://doi.org/10.2307/j.ctv301g2x

Eberhard, S.M., Smith, G.B., Gibian, M.M., Smith, H.M., Gray, M.R., 2014. Invertebrate Cave Fauna of Jenolan. Proc. Linn. Soc. NSW 136, 35–67. https://doi.org/10.1109/FUZZ-IEEE.2017.8015520

Engler-Chaouat, H.S., Gilbert, L.E., 2007. De novo Synthesis vs . Sequestration : Negatively Correlated Metabolic Traits and the Evolution of Host Plant Specialization in Cyanogenic Butterflies. J. Chem. Ecol. 33, 25–42. https://doi.org/10.1007/s10886-006-9207-8

Espeland, M., Breinholt, J., Willmott, K.R., Warren, A.D., Vila, R., Toussaint, E.F.A., Maunsell, S.C., Aduse-Poku, K., Talavera, G., Eastwood, R., Jarzyna, M.A., Guralnick, R., Lohman, D.J., Pierce, N.E., Kawahara, A.Y., 2018. A Comprehensive and Dated Phylogenomic Analysis of Butterflies. Curr. Biol. https://doi.org/10.1016/j.cub.2018.01.061

Fenton, M.B., Gaudet, C.L., Leonard, M.L., 1983. Feeding behaviour of the bats Nycteris grandis and Nycteris thebaica (Nycteridae) in captivity. J. Zool. 200, 347–354. https://doi.org/10.1111/j.1469-7998.1983.tb02315.x

Fenton, M.B., Roeder, K.D., 1974. The microtymbals of some Arctiidae. J. Lepid. Soc. 28, 205–211.

Firzlaff, U., Schuchmann, M., Grunwald, J.E., Schuller, G., Wiegrebe, L., 2007. Object-oriented echo perception and cortical representation in echolocating bats. PLoS Biol. 5, 1174–1183. https://doi.org/10.1371/journal.pbio.0050100

Fullard, J.H., 1994. Auditory changes in noctuid moths endemic to a bat-free habitat. J. Evol. Biol. 7, 435–445.

Fullard, J.H., 1988. The tuning of moth ears. Experientia 44, 423–428.

Fullard, J.H., Heller, B., 1990. Functional Organization of the Arctiid Moth Tymbal (Insecta, Lepidoptera). J. Morphol. 204, 57–65. https://doi.org/10.1002/jmor.1052040107

Fullard, J.H., Napoleone, N., 2001. Diel flight periodicity and the evolution of auditory defences in the Macrolepidoptera. Anim. Behav. 62, 349–368. https://doi.org/10.1006/anbe.2001.1753

Fullard, J.H., Ratcliffe, J.M., Soutar, A.R., 2004. Extinction of the acoustic startle

response in moths endemic to a bat-free habitat. J. Evol. Biol. 17, 856–861. https://doi.org/10.1111/j.1420-9101.2004.00722.x

Fullard, J.H., Yack, J.E., 1993. The evolutionary biology of insect hearing. Trends Ecol. Evol. 8, 248–252. https://doi.org/10.1016/0169-5347(93)90200-9

Fung, S.Y., Herrebout, W.M., Verpoorte, R., Fischer, F.C., 1988. Butenolides in small ermine moths, Yponomeuta spp. (Lepidoptera: Yponomeutidae), and spindle-tree, Euonymus europaeus (Celastraceae). J. Chem. Ecol. 14, 1099–1111. https://doi.org/10.1007/BF01019338

Gamania, N., Uuugi, J., n.d. JPMoth.org [WWW Document]. URL http://jpmoth.org/ (accessed 10.18.17).

Goerlitz, H.R., Ter Hofstede, H.M., Zeale, M.R.K., Jones, G., Holderied, M.W., 2010. An aerial-hawking bat uses stealth echolocation to counter moth hearing. Curr. Biol. 20, 1568–1572. https://doi.org/10.1016/j.cub.2010.07.046

Gonsalves, L., Bicknell, B., Law, B., Webb, C., Monamy, V., 2013. Mosquito Consumption by Insectivorous Bats: Does Size Matter? PLoS One 8, 1–11. https://doi.org/10.1371/journal.pone.0077183

Göpfert, M.C., Wasserthal, L.T., 1999. Hearing with the mouthparts: behavioural responses and the structural basis of ultrasound perception in acherotiine hawkmoths. J. Exp. Biol. 202, 909–918.

Götz, T., Janik, V.M., 2011. Repeated elicitation of the acoustic startle reflex leads to sensitisation in subsequent avoidance behaviour and induces fear conditioning. BMC Neurosci. 12, 1–12. https://doi.org/10.1186/1471-2202-12-30

Greenfield, M.D., 2016. Evolution of Acoustic Communication in Insects, in: Pollack, G.S., Mason, A.C., Popper, A.N., Fay, R.R. (Eds.), Insect Hearing. Springer International Publishing, Cham, pp. 17–47. https://doi.org/10.1007/978-3-319-28890-1_2

Greenfield, M.D., 2014. Acoustic Communication in the Nocturnal Lepidoptera, in: Hedwig, B. (Ed.), Insect Hearing and Acoustic Communication. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 81–100. https://doi.org/10.1007/978-3-642-40462-7_6

Gwynne, D.T., Edwards, E.D., 1986. Ultrasound production by genital stridulation in Syntonarcha iriastis (Lepidoptera: Pyralidae): long-distance signalling by male moths? Zool. J. Linn. Soc. 88, 363–376.

Hamilton-Smith, E., 1967. The Arthropoda of Australian Caves. J. Aust. Entomol. Soc. 6, 103–118.

Hay-Roe, M.M., Mankin, R.W., 2004. Wing-click sounds of Heliconius cydno alithea (Nymphalidae: Heliconiinae) butterflies. J. Insect Behav. 17, 329–335. https://doi.org/10.1023/B:JOIR.0000031534.18848.c1

Heffner, H.E., Heffner, R.S., 2007. Hearing ranges of laboratory animals. J. Am. Assoc. Lab. Anim. Sci. 46, 20–2.

Heller, K., Achmann, R., 1993. The ultrasonic song of the moth Amyna natalis (Lepidoptera: Noctuidae: Acontiinae). Bioacoustics 5, 89–97.

Heppner, J.B., 2008. Ermine Moths (Lepidoptera: Yponomeutidae), in: Capinera, J.L. (Ed.), Encyclopedia of Entomology. Springer Netherlands, Dordrecht, pp. 1360–1361. https://doi.org/10.1007/978-1-4020-6359-6_3661

Holderied, M., Korine, C., Moritz, T., 2011. Hemprich's long-eared bat (Otonycteris hemprichii) as a predator of scorpions: Whispering echolocation, passive gleaning and prey selection. J. Comp. Physiol. A 197, 425–433. https://doi.org/10.1007/s00359-010-0608-3

Holderied, M.W., Korine, C., Fenton, M.B., Parsons, S., Robson, S., Jones, G., 2005. Echolocation call intensity in the aerial hawking bat Eptesicus bottae (Vespertilionidae) studied using stereo videogrammetry. J. Exp. Biol. 208, 1321–1327. https://doi.org/10.1242/jeb.01528

Holderied, M.W., Thomas, L.A., Korine, C., 2018. Ultrasound avoidance by flying antlions (Myrmeleontidae). J. Exp. Biol. 221, 1–6. https://doi.org/10.1242/jeb.189308

Howarth, F.G., 1980. The zoogeography of specialized cave animals: a bioclimatic model. Evolution (N. Y). 34, 394–406.

Hoy, R.R., Robert, D., 1996. Tympanal Hearing in Insects. Annu. Rev. Entomol. 41, 433–450. https://doi.org/10.1146/annurev.ento.41.1.433

Hristov, N.I., Conner, W.E., 2005a. Effectiveness of tiger moth (Lepidoptera, Arctiidae) chemical defenses against an insectivorous bat (Eptesicus fuscus). Chemoecology 15, 105–113. https://doi.org/10.1007/s00049-005-0301-0

Hristov, N.I., Conner, W.E., 2005b. Sound strategy: acoustic aposematism in the bat-tiger moth arms race. Naturwissenschaften 92, 164–169. https://doi.org/10.1007/s00114-005-0611-7

Hu, N., Burgueño, R., 2015. Buckling-induced smart applications: recent advances and trends. Smart Mater. Struct. 24. https://doi.org/10.1088/0964-1726/24/6/063001

Huang, G.-H., Chen, L.-S., Hirowatari, T., Nasu, Y., Wang, M., 2011. A revision of the Monopis monachella species complex (Lepidoptera: Tineidae) from China. Zool. J. Linn. Soc. 163, 1–14. https://doi.org/10.1111/j.1096-3642.2011.00704.x

Humphreys, W.F., Eberhard, S., 2001. Subterranean Fauna of Christmas Island , Indian Ocean. Helictite 37, 59–74.

Jakšić, P., 2017. Cave moth and butterfly fauna (Insecta: Lepidoptera) of Serbia: Current state and future prospects. Univ. Thought - Publ. Nat. Sci. 7, 8–12. https://doi.org/10.5937/univtho7-14038

Jang, Y., Greenfield, M.D., 1996. Ultrasonic communication and sexual selection in waxmoths: female choice based on energy and asynchrony of male signals. Anim. Behav. 51, 1095–1106. https://doi.org/https://doi.org/10.1006/anbe.1996.0111

Jones, G., Teeling, E.C., 2006. The evolution of echolocation in bats. Trends Ecol. Evol. 21, 149–156.

Juliana, J.R. St., Fenton, B.M., Korine, C., Pinshow, B., Wojciechowski, M., Kravchenko, V., 2007. Note: A Field Assessment of the Defensive Responses of Moths to an Auditory Stimulus. Isr. J. Ecol. Evol. 53, 173–177. https://doi.org/10.1560/IJEE.53.2.173

Kawahara, A.Y., Barber, J.R., 2015. Tempo and mode of antibat ultrasound production and sonar jamming in the diverse hawkmoth radiation. Proc. Natl. Acad. Sci. 112, 6407–6412. https://doi.org/10.1073/pnas.1416679112

Kawahara, A.Y., Plotkin, D., Hamilton, C.A., Gough, H., St Laurent, R., Owens, H.L., Homziak, N.T., Barber, J.R., 2017. Diel behavior in moths and butterflies: a synthesis of data illuminates the evolution of temporal activity. Org. Divers. Evol. 1–15. https://doi.org/10.1007/s13127-017-0350-6

Kingsolver, J.G., 1983. Thermoregulation and Flight in Colias Butterflies : Elevational Patterns and Mechanistic Limitations Author (s): Joel G . Kingsolver Published by : Wiley on behalf of the Ecological Society of America Stable URL : http://www.jstor.org/stable/1939973 REF. Ecology 64, 534–545.

Koay, G., Heffner, H.E., Heffner, R.S., 1997. Audiogram of the big brown bat (Eptesicus fuscus). Hear. Res. 105, 202–210. https://doi.org/10.1016/S0378-5955(96)00208-0

Kovalev, I.S., 2016. Stealth Moths: The Multi-Plumed Wings of the Moth Alucita hexadactyla May Decrease the Intensity of Their Echo to Simulated Bat Echolocation Cries. Entomol. News 126, 204–212.

Kumar, S., Hedges, S.B., 1998. A molecular timescale for vertebrate evolution. Nature 392, 917–920.

Landsberg, H., 1954. Bioclimatology of Housing, in: Sargent, F., Stone, R.G. (Eds.), Recent Studies in Bioclimatology: A Group. American Meteorological Society, Boston, MA, pp. 81–98. https://doi.org/10.1007/978-1-940033-11-2_9

Larsson, S., Tenow, O., 1980. Needle-eating insects and grazing dynamics in a mature Scots pine forest in central Sweden. Oikos 32, 269–306.

László, R., 2004. Lepidoptere din peşterile României. Bul. Inf. Entomol. 14–15, 201–206.

Lee, D.J., Ju, Y.D., Bayarsaikhan, U., Park, B.S., Na, S.M., Kim, J.W., Lee, B.W., Bae, Y.S., 2016. First report on two species of genus Monopis (Lepidoptera, Tineidae) collected by feather trap in Korea. J. Asia-Pacific Biodivers. 9, 215–218. https://doi.org/10.1016/j.japb.2016.02.007

Lee, W., Moss, C.F., 2016. Can the elongated hindwing tails of fluttering moths serve as false sonar targets to divert bat attacks ? J. Acoust. Soc. Am. 139, 2579–2588. https://doi.org/10.1121/1.4947423

Lees, D.C., 1992. Foreleg stridulation in male Urania moths (Lepidoptera: Uraniidae). Zool. J. Linn. Soc. 106, 163–170.

Lucas, K.M., Mongrain, J.K., Windmill, J.F.C., Robert, D., Yack, J.E., 2014. Hearing in the crepuscular owl butterfly (Caligo eurilochus, Nymphalidae). J. Comp. Physiol. A Neuroethol. Sensory, Neural, Behav. Physiol. https://doi.org/10.1007/s00359-014-0933-z

Marsh, N., Rothschild, M., 1974. Aposematic and cryptic Lepidoptera tested on the mouse. J. Zool. 174, 89–122. https://doi.org/10.1111/j.1469-7998.1974.tb03146.x

Menken, S.B.J., Heerebout, W.M., Wiebes, J.T., 1992. Small ermine moths (Yponomeuta): Their Host Relations and Evolution. Annu. Rev. Entomol. 37, 41–66.

Metz, M.A., Davis, D.R., Davis, M.M., 2018. A New Species of Niditinea (Tineidae: Tineinae) with a Preference for Bird Nests, and the Known Larval Habitats of the Species in the United States . Proc. Entomol. Soc. Washingt. 120, 153–166. https://doi.org/10.4289/0013-8797.120.1.153

Meyrick, E., 1895. A handbook of British Lepidoptera. London,Macmillan.

Miller, L.A., Surlykke, A., 2001. How some insects detect and avoid being eaten by bats: tactics and countertactics of prey and predator. Bioscience 51, 570–581.

Møhl, B., 1988. Target Detection by Echolocating Bats, in: Nachtigall, P.E., Moore, P.W.B. (Eds.), Animal Sonar: Processes and Performance. Springer US, Boston, MA, pp. 435–450. https://doi.org/10.1007/978-1-4684-7493-0_43

Monge-Nájera, J., Hernández, F., González, M.I., Soley, J., Araya, J., Zolla, S., 1998. Spatial distribution, territoriality and sound production by tropical cryptic butterflies (Hamadryas, Lepidoptera: Nymphalidae): implications for the “industrial melanism” debate. Rev. Biol. Trop. 46, 297–330.

Mosconi, F., 2011. Biologia comparata dei princpali Lepidotteri cavernicoli Italiani nella loro ecofase sotterranea. Sapienza Università di Roma Corso di Dottorato in Biologia Animale XXII Ciclo.

Müller, F., 1879. Ituna and Thyridia: a remarkable case of mimicry in butterflies.

Trans. Entomol. Soc. London xx–xxix. https://doi.org/10.1017/CBO9781107415324.004

Nakano, R., Skals, N., Takanashi, T., Surlykke, A., Koike, T., Yoshida, K., Maruyama, H., Tatsuki, S., Ishikawa, Y., 2008. Moths produce extremely quiet ultrasonic courtship songs by rubbing specialized scales. Proc. Natl. Acad. Sci. U. S. A. 105, 11812–11817. https://doi.org/10.1073/pnas.0804056105

Nakano, R., Takanashi, T., Fujii, T., Skals, N., Surlykke, A., Ishikawa, Y., 2009. Moths are not silent, but whisper ultrasonic courtship songs. J. Exp. Biol. 212, 4072–4078. https://doi.org/10.1242/jeb.032466

Nakano, R., Takanashi, T., Surlykke, A., Skals, N., Ishikawa, Y., 2013. Evolution of deceptive and true courtship songs in moths. Sci. Rep. 3, 2003. https://doi.org/10.1038/srep02003

Neil, T.R., Shen, Z., Drinkwater, B.W., Robert, D., Holderied, M.W., 2018. Stealthy moths avoid bats with acoustic camouflage. J. Acoust. Soc. Am. 144, 1742. https://doi.org/10.1121/1.5067725

Nentwig, W., 1982. Why Do Only Certain Insects Escape from a Spider's Web? Oecologia 53, 412–417.

Neuweiler, G., Singh, S., Sripathi, K., 1984. Audiograms of a South Indian bat community. J. Comp. Physiol. A 154, 133–142. https://doi.org/10.1007/BF00605398

Ntelezos, A., Guarato, F., Windmill, J.F.C., 2017. The anti-bat strategy of ultrasound absorption: the wings of nocturnal moths (Bombycoidea: Saturniidae) absorb more ultrasound than the wings of diurnal moths (Chalcosiinae: Zygaenoidea: Zygaenidae). Biol. Open 6, 109–117. https://doi.org/10.1242/bio.021782

O'Reilly, L.J., Agassiz, D.J.L., Neil, T.R., Holderied, M.W., 2019. Deaf moths employ acoustic Müllerian mimicry against bats using wingbeat-powered tymbals. Sci. Rep. 9, 1–9. https://doi.org/10.1038/s41598-018-37812-z

Pape, R.B., 2014. Biology and ecology of Bat cave, Grand Canyon National Park, Arizona. J. Cave Karst Stud. 76, 1–13. https://doi.org/10.4311/2012LSC0266

Pass, G.J., Foley, W.J., 2000. Plant secondary metabolites as mammalian feeding

deterrents: separating the effects of the taste of salicin from its post-ingestive consequences in the common brushtail possum (Trichosurus vulpecula). J. Comp. Physiol. B 170, 185–192. https://doi.org/10.1007/s003600050274

Peck, S.B., 1975. The invertebrate fauna of tropical American caves, Part III: Jamaica, an introduction. Int. J. Speleol. 7, 303–326. https://doi.org/http://dx.doi.org/10.5038/1827-806X.7.4.1

Peck, S.B., 1974. The Invertebrate Fauna of Tropical American Caves , Part II : Puerto Rico , An Ecological and Zoogeographic Analysis The Invertebrate Fauna of Tropical American Caves , Part II : Puerto Rico , An Ecological and Zoogeographic Analysis. Biotropica 6, 14–31.

Pickering, T.R., Egeland, C.P., Domínguez-Rodrigo, M., Brain, C.K., Schnell, A.G., 2008. Testing the "shift in the balance of power" hypothesis at Swartkrans, South Africa: Hominid cave use and subsistence behavior in the Early Pleistocene. J. Anthropol. Archaeol. 27, 30–45. https://doi.org/10.1016/j.jaa.2007.07.002

Polak, S., Bedek, J., Ozimec, R., 2012. Subterranean fauna of twelve istrian caves. Ann. Ser. Hist. Nat. 22, 7–24.

Pringle, J.W.S., 1937. Proprioreception in insects. J. Exp. Biol. 15, 101–113.

Pye, J.D., 1993. Is fidelity futile? The "true" signal is illusory, especially with ultrasound. Bioacoustics 4, 271–286. https://doi.org/10.1080/09524622.1993.10510438

Ratcliffe, J.M., 2009. Predator-Prey Interaction in an Auditory World, in: Dukas, R., Ratcliffe, J.M. (Eds.), Cognitive Ecology II. University of Chicago Press, pp. 201–228. https://doi.org/10.1016/j.anbehav.2010.03.021

Ratcliffe, J.M., Nydam, M.L., 2008. Multimodal warning signals for a multiple predator world. Nature 455, 96.

Ratnasingham, S., Hebert, P.D.N., 2007. bold: The Barcode of Life Data System (http://www.barcodinglife.org). Mol. Ecol. Notes 7, 355–364. https://doi.org/10.1111/j.1471-8286.2006.01678.x

Regier, J.C., Mitter, C., Zwick, A., Bazinet, A.L., Cummings, M.P., Kawahara, A.Y.,

Sohn, J.C., Zwickl, D.J., Cho, S., Davis, D.R., Baixeras, J., Brown, J., Parr, C., Weller, S., Lees, D.C., Mitter, K.T., 2013. A Large-Scale, Higher-Level, Molecular Phylogenetic Study of the Insect Order Lepidoptera (Moths and Butterflies). PLoS One 8. https://doi.org/10.1371/journal.pone.0058568

Robinson, G.S., 1980. Cave-dwelling Tineid Moths: a Taxonomic Review of the World Species (Lepidoptera: Tineidae). Trans. Br. Cave Res. Assoc. 7, 83–120.

Robinson, G.S., 1979. Clothes-moths of the Tinea pellionella complex: a revision of the world's species (Lepidoptera: Tineidae). Bull. Br. Museum (Natural Hist. Entomol. 38, 57–128.

Roeder, D., 1962. Echoes of ultrasonic pulses from flying moths. Biol. Bull. 124, 200–210.

Roeder, K.D., 1964. Aspects of the noctuid tympanic nerve response having significance in the avoidance of bats. J. Insect Physiol. 10, 529–546. https://doi.org/10.1016/0022-1910(64)90025-3

Roeder, K.D., Treat, A.E., 1957. Ultrasonic reception by the tympanic organ of noctuid moths. J. Exp. Zool. 134, 127–157. https://doi.org/10.1002/jez.1401340107

Roessingh, P., Hora, K.H., Fung, S.Y., Peltenburg, A., Menken, S.B.J., 2000. Host acceptance behaviour of the small ermine moth Yponomeuta cagnagellus: larvae and adults use different stimuli. Chemoecology 10, 41–47. https://doi.org/10.1007/s000490050006

Rothschild, M., Reichstein, T., von Euw, J., Aplin, R., Harman, R.R.M., 1970. Toxic Lepidoptera. Toxicon 8, 293–299. https://doi.org/10.1016/0041-0101(70)90006-1

Rowe, C., Halpin, C., 2013. Why are warning displays multimodal? Behav. Ecol. Sociobiol. 67, 1425–1439. https://doi.org/10.1007/s00265-013-1515-8

Russo, D., Maglio, G., Rainho, A., Meyer, C.F.J., Palmeirim, J.M., 2011. Out of the dark : Diurnal activity in the bat Hipposideros ruber on São Tomé island (West Africa) 76, 701–708. https://doi.org/10.1016/j.mambio.2010.11.007

Rydell, J., 1998. Bat defence in lekking ghost swifts (Hepialus humuli), a moth

without ultrasonic hearing. Proc. R. Soc. B Biol. Sci. 265, 1373–1376. https://doi.org/10.1098/rspb.1998.0444

Sargison, N.D., Williamson, D.S., Duncan, J.R., McCance, R.W., 1996. Prunus padus (bird cherry) poisoning in cattle. Vet. Rec. 138, 188. https://doi.org/citeulike-article-id:13693721

Shen, Z., Neil, T.R., Robert, D., Drinkwater, B.W., Holderied, M.W., 2018. Biomechanics of a moth scale at ultrasonic frequencies. Proc. Natl. Acad. Sci. 115, 12200–12205. https://doi.org/10.1073/pnas.1810025115

Sillar, K.T., Picton, L.D., Heitler, W.J., 2016. The Mammalian Startle Response. Neuroethol. Predation Escape, Wiley Online Books. https://doi.org/doi:10.1002/9781118527061.ch9

Silva, M.S., Ferreira, R.L., 2015. Cave invertebrates in Espírito Santo state, Brazil: A primary analysis of endemism, threats and conservation priorities. Subterr. Biol. 16, 79–102. https://doi.org/10.3897/subtbiol.16.5227

Simmons, J.A., Howell, D.J., Suga, N., 1975. Information Content of Bat Sonar Echoes. Am. Sci. 63, 204–215.

Simmons, R.B., Conner, W.E., 1996. Ultrasonic signals in the defense and courtship of Euchaetes egle Drury and E. bolteri Stretch (Lepidoptera: Arctiidae). J. Insect Behav. 9, 909–919. https://doi.org/10.1007/BF02208978

Skals, N., Surlykke, A., 2000. Hearing and evasive behaviour in the greater wax moth, Galleria mellonella (Pyralidae). Physiol. Entomol. 25, 354–362. https://doi.org/10.1046/j.1365-3032.2000.00204.x

Skals, N., Surlykke, A., 1999. Sound production by abdominal tymbal organs in two moth species: the green silver-line and the scarce silver-line (Noctuoidea: Nolidae: Chloephorinae). J. Exp. Biol. 202, 2937–2949.

Slegers, N., Heilman, M., Cranford, J., Lang, A., Yoder, J., Habegger, M.L., 2017. Beneficial aerodynamic effect of wing scales on the climbing flight of butterflies. Bioinspiration and Biomimetics 12. https://doi.org/10.1088/1748-3190/aa551d

Slootmaekers, D., 2013. Infurcitinea ignicomella (Lepidoptera: Tineidae, Meessiinae),

new to the Belgian fauna. Phegea 41, 17–18.

Sohn, J.C., 2016. Review of the genus Eumonopyta (Lepidoptera: Yponomeutidae) with descriptions of two new species. Entomol. Sci. 19, 155–160. https://doi.org/10.1111/ens.12188

Sohn, J.C., Regier, J.C., Mitter, C., Davis, D., Landry, J.F., Zwick, A., Cummings, M.P., 2013. A Molecular Phylogeny for Yponomeutoidea (Insecta, Lepidoptera, Ditrysia) and Its Implications for Classification, Biogeography and the Evolution of Host Plant Use. PLoS One 8. https://doi.org/10.1371/journal.pone.0055066

Spangler, H.G., 1984. Silence as a defense against predatory bats in two species of calling insects. Southwest. Nat. 29, 481–488.

Stevens, R.E., 1971. Pine needle-sheath miner. USDA FS For. Pest Leafl. 65, 1–5.

Stringer, I.A.N., Meyer-Rochow, V.B., 1997. Flight activity of insects within a Jamaican cave: in search of the zeitgeber. Invertebr. Biol. 116, 348–354.

Sun, J., Bhushan, B., 2012. Structure and mechanical properties of beetle wings: A review. RSC Adv. 2, 12606–12623. https://doi.org/10.1039/c2ra21276e

Surlykke, A., 1984. Hearing in notodontid moths: a tympanic organ with a single auditory neurone. J. Exp. Biol. 113, 323–335.

Surlykke, A., Gogala, M., 1986. Stridulation and hearing in the noctuid moth Thecophora fovea (Tr.). J. Comp. Physiol. A 159, 267–273. https://doi.org/10.1007/BF00612309

Surlykke, A., Miller, L.A., 1985. The influence of arctiid moth clicks on bat echolocation; jamming or warning ? J. Comp. Physiol. A 156, 831–843.

Takács, S., Mistal, C., Gries, G., 2003. Communication ecology of webbing clothes moth: Attractiveness and characterization of male-produced sonic aggregation signals. J. Appl. Entomol. 127, 127–133. https://doi.org/10.1046/j.1439-0418.2003.00724.x

Tawara, J.N., Blokhin, A., Foderaro, T.A., Stermitz, F.R., Hope, H., 1993. Toxic Piperidine Alkaloids from Pine (Pinus) and Spruce (Picea) Trees. New Structures and a Biosynthetic Hypothesis. J. Org. Chem. 58, 4813–4818.

https://doi.org/10.1021/jo00070a014

ter Hofstede, H.M., Goerlitz, H.R., Ratcliffe, J.M., Holderied, M.W., Surlykke, A., 2013. The simple ears of noctuoid moths are tuned to the calls of their sympatric bat community. J. Exp. Biol. 216, 3954–3962. https://doi.org/10.1242/jeb.093294

ter Hofstede, H.M., Ratcliffe, J.M., 2016. Evolutionary escalation : the bat – moth arms race. J. Exp. Biol. 219, 1589–1602. https://doi.org/10.1242/jeb.086686

Trajano, E., 2000. Cave Faunas in the Atlantic Tropical Rain Forest : Composition , Ecology , and Conservation. Biotropica 32, 882–893.

Turbanov, I.S., Palatov, D.M., Golovatch, S.I., 2016. The state of the art of biospeleology in Russia and other countries of the former Soviet Union: A review of the cave (endogean) invertebrate fauna. 3. References. Entomol. Rev. 96, 1297–1333. https://doi.org/10.1134/S0013873816090128

Turner, H., Lieshout, N., Van Ginkel, W.E., Menken, S.B.J., 2010. Molecular phylogeny of the small ermine moth genus Yponomeuta (Lepidoptera, Yponomeutidae) in the Palaearctic. PLoS One 5, 15–19. https://doi.org/10.1371/journal.pone.0009933

Van Nieukerken, E.J., Kaila, L., Kitching, I.J., Kristensen, N.P., Lees, D.C., Minet, J., Mitter, C., Mutanen, M., Regier, J.C., 2011. Order Lepidoptera Linnaeus, 1758. In: Zhang, Z.-Q. (Ed.), Animal biodiversity: An outline of higher-level classification and survey of taxonomic richness. Zootaxa 3148, 212–221. https://doi.org/http://www.mapress.com/zootaxa/2011/f/zt03148p221.pdf

Vukusic, P., Sambles, J.R., 2003. Photonic structures in biology. Nature 424, 852–855.

Wagner, H., Flitsch, K., Jurcic, K., 1981. Cytotoxizität von Siphonosid und aliphatischen Estern des Siphonodins. Planta Med. 43, 249–251. https://doi.org/10.1055/s-2007-971505

Wahlberg, N., Wheat, C.W., Peña, C., 2013. Timing and patterns in the taxonomic diversification of Lepidoptera (butterflies and moths). PLoS One 8, 1–8. https://doi.org/10.1371/journal.pone.0080875

Waters, D.A., Rydell, J., Jones, G., 1995. Echolocation call design and limits on prey size: a case study using the aerial-hawking bat Nyctalus leisleri. Behav. Ecol. Sociobiol. 37, 321–328. https://doi.org/10.1007/BF00174136

Watson, L., Dallwitz, M., n.d. Insects of Britain and Ireland: the genera of grass moths (Pyralidae- Crambidae and Schoenobiinae), Version: 12th February 2019. [WWW Document]. URL delta-intkey.com (accessed 5.5.19).

Weller, S.J., Jacobson, N.L., Connor, W.E., 1999. The evolution of chemical defenses and mating systems in tiger moths (Lepidoptera: Arctiidae). Biol. J. Linn. Soc. 68, 557–578. https://doi.org/10.1111/j.1095-8312.1999.tb01188.x

Wootton, R.J., 1979. Function, homology and terminology in insect wings. Syst. Entomol. 4, 81–93. https://doi.org/10.1111/j.1365-3113.1979.tb00614.x

Wynne, J.J., Drost, C.A., Cobb, N.S., Rihs, J.R., 2005. Cave-Dwelling Invertebrates of Grand Canyon National Park.

Wynne, J.J., Pleytez, W., 2005. Sensitive ecological areas and species inventory of Actun Chapat Cave, Vaca Plateau, Belize. J. Cave Karst Stud. 67, 148–157.

Xiao, Y.L., Li, H.H., 2005. A systematic study on the genus Crypsithyris Meyrick, 1907 from China (Lepidoptera: Tineidae). Shil. Rev. Lepidopterol. 33, 17–23.

Yack, J.E., 1988. Seasonal partitioning of atympanate moths in relation to bat activity. Can. J. Zool. 66, 753–755. https://doi.org/10.1139/z88-111

Yack, J.E., Kalko, E.K. V, Surlykke, A., 2007. Neuroethology of ultrasonic hearing in nocturnal butterflies (Hedyloidea). J. Comp. Physiol. A 193, 577–590. https://doi.org/10.1007/s00359-007-0213-2

Yack, J.E., Otero, D.L., Dawson, J.W., Surlykke, A., Fullard, J.H., 2000. Sound production and hearing in the blue cracker butterfly Hamadryas feronia (Lepidoptera, Nymphalidae) from Venezuela. J. Exp. Biol. 203, 3689–3702.

Yager, D.D., Spangler, H.G., 1997. Behavioural response to ultrasound by the tiger beetle Cincindela amrutha Dow combines aerodynamic changes and sound production. J. Exp. Biol. 200, 649–659.

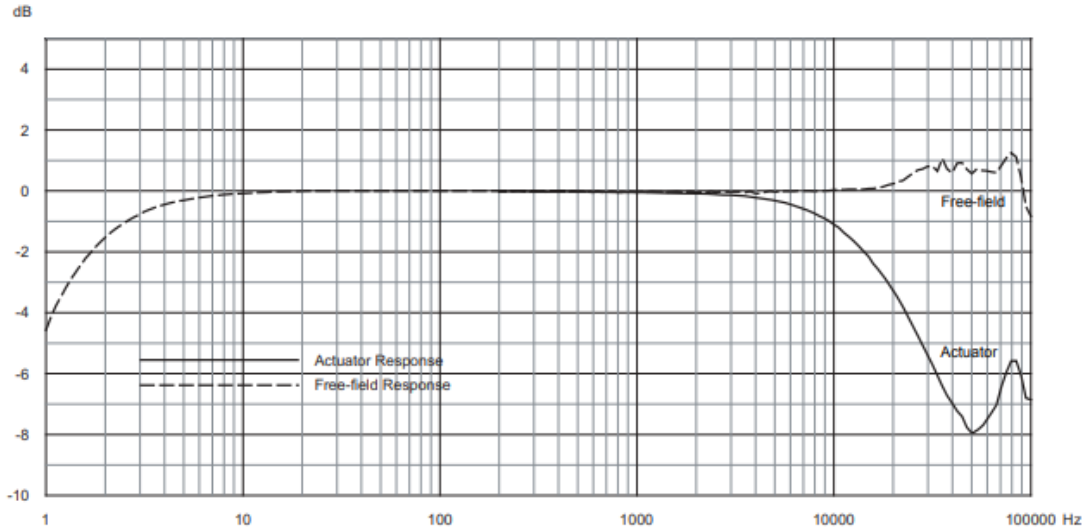
Yang, X.-S., 2010. A New Metaheuristic Bat-Inspired Algorithm, in: González, J.R.,

Pelta, D.A., Cruz, C., Terrazas, G., Krasnogor, N. (Eds.), Nature Inspired Cooperative Strategies for Optimization (NICSO 2010). Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 65–74. https://doi.org/10.1007/978-3-642-12538-6_6

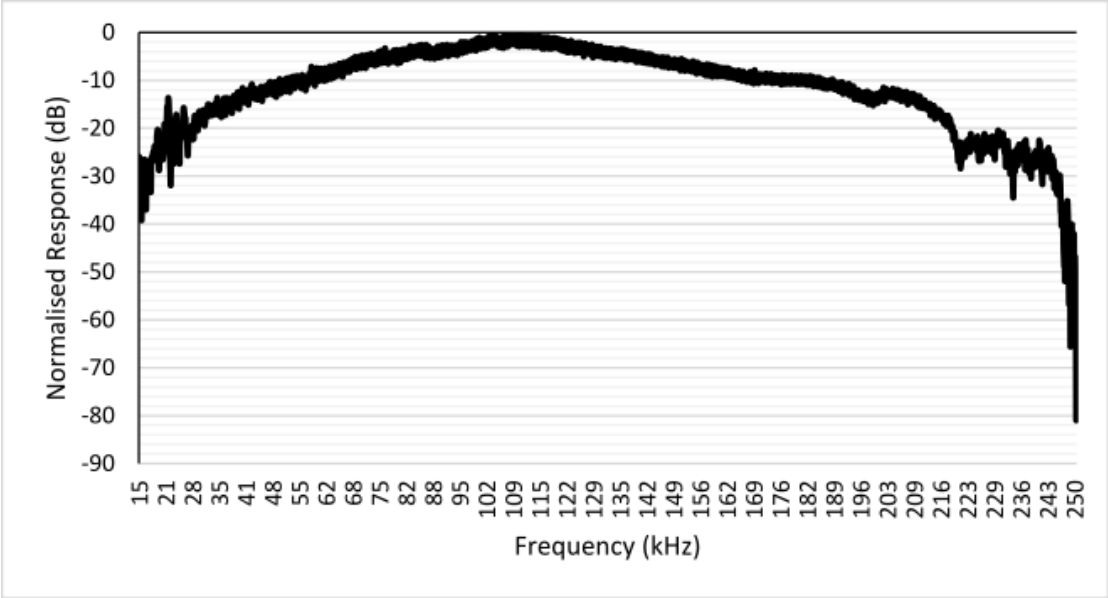
Young, D., Bennet-Clark, H., 1995. The role of the tymbal in cicada sound production. J. Exp. Biol. 198, 1001–1019.

Zeng, J., Xiang, N., Jiang, L., Jones, G., Zheng, Y., Liu, B., Zhang, S., 2011. Moth wing scales slightly increase the absorbance of bat echolocation calls. PLoS One 6. https://doi.org/10.1371/journal.pone.0027190

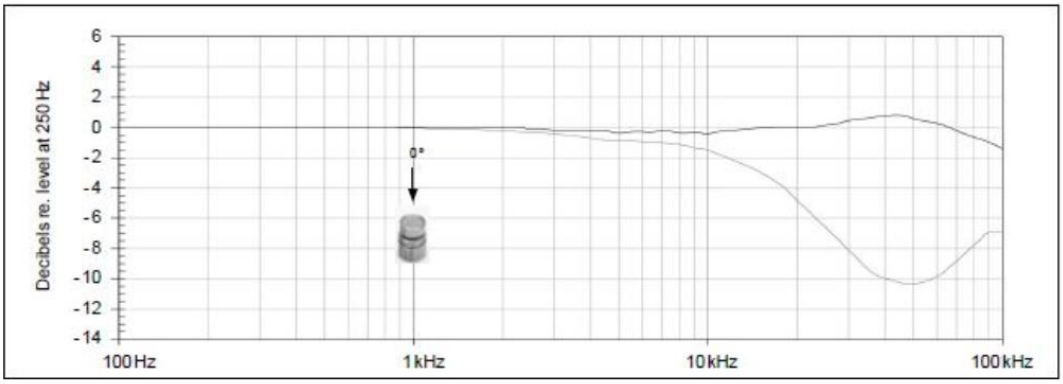
Appendices



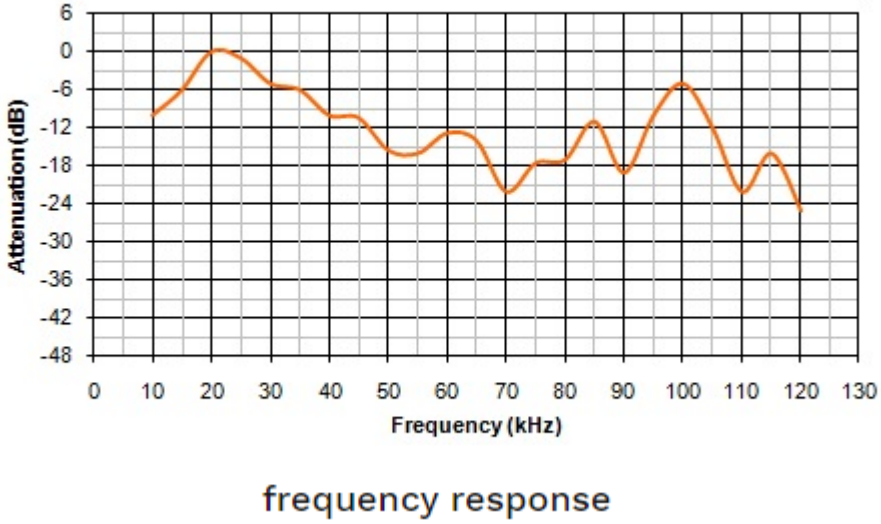
Appendix 1 Frequency response of ¼" Brüel and Kjær freefield microphone taken from their product specifications.



Appendix 2 Frequency response of the custom-made ferro-electret foil loudspeaker used in acoustic tomography measurements.



Appendix 3 Frequency response (black line) of GRAS 40BE ¼" polarised free-field microphone, taken from the product specifications.



Appendix 4 Frequency response of Avisoft Bioacoustics USG Omnidirectional Electret Ultrasound Knowles FG-O microphone, taken from the product specifications.

Appendix 5 Regression equations and R squared values for linear regressions of echo intensity (target strength) and log10 area of lepidopteran left wings from Chapter 4. All data were split into 5 kHz frequency bands between 20 and 100 kHz prior to running regressions.

Frequency Band (kHz)	Equation	R^2
20-25	y = 16.791x – 47.021	0.6986
25-30	y = 15.456x – 42.895	0.6529
30-35	y = 14.926x – 41.234	0.6157
35-40	y = 14.626x – 40.476	0.5746
40-45	y = 14.494x – 40.304	0.5579
45-50	y = 14.301x – 40.09	0.5531
50-55	y = 14.059x – 39.936	0.5358
55-60	y = 13.7x – 39.609	0.5113
60-65	y = 13.396x – 39.533	0.48
65-70	y = 13.468x – 40.507	0.452
70-75	y = 13.857x – 42.377	0.4332
75-80	y = 14.186x – 44.233	0.418
80-85	y = 14.415x – 45.924	0.3977
85-90	y = 14.875x – 48.212	0.3864
90-95	y = 15.197x – 50.122	0.3827
95-100	y = 15.617x – 52.262	0.3907

Appendix 6 Regression equations and R squared values for linear regressions of echo intensity (target strength) and log10 area of lepidopteran bodies from Chapter 4. All data were split into 5 kHz frequency bands between 20 and 100 kHz prior to running regressions.

Frequency Band (kHz)	Equation	R^2
20-25	y = 9.3326x-41.203	0.3334
25-30	y = 8.625x-39.902	0.2721
30-35	y = 8.4542x-39.714	0.2456
35-40	y = 8.4975x-39.95	0.2326
40-45	y = 8.2349x-39.694	0.21
45-50	y = 7.9126x-39.325	0.195
50-55	y = 7.6483x-39.163	0.1838
55-60	y = 7.4919x-39.393	0.1737
60-65	y = 7.4892x-40.001	0.1597
65-70	y = 7.8222x-41.268	0.1558
70-75	y = 7.9235x-42.212	0.1507
75-80	y = 8.5497x-44.06	0.1665
80-85	y = 9.1463x-46.059	0.1739w
85-90	y = 9.5616x-47.759	0.1771
90-95	y = 10.257x-49.897	0.1859
95-100	y = 10.927x-51.981	0.2003

Appendix 7 Regression equations and R squared values for linear regressions of echo intensity (target strength) and log10 area of moth wings from Chapter 4. All data were split into 5 kHz frequency bands between 20 and 100 kHz prior to running regressions.

Frequency Band (kHz)	Equation	R^2
20-25	y = 16.864x-47.994	0.7012
25-30	y = 15.421x-43.683	0.6532
30-35	y = 14.525x-41.205	0.5963
35-40	y = 13.851x-39.621	0.539
40-45	y = 13.385x-38.72	0.5158
45-50	y = 13.096x-38.34	0.5146
50-55	y = 12.986x-38.554	0.5051
55-60	y = 12.586x-38.2	0.489
60-65	y = 12.107x-37.839	0.4592
65-70	y = 12.085x-38.726	0.4291
70-75	y = 12.427x-40.595	0.4163
75-80	y = 12.56x-42.084	0.4028
80-85	y = 12.545x-43.317	0.3793
85-90	y = 13.044x-45.71	0.3682
90-95	y = 13.462x-47.764	0.3656
95-100	y = 13.956x-49.996	0.3747

Appendix 8 Regression equations and R squared values for linear regressions of echo intensity (target strength) and log10 area of moth bodies from Chapter 4. All data were split into 5 kHz frequency bands between 20 and 100 kHz prior to running regressions.

Frequency Band (kHz)	Equation	R^2
20-25	y = 9.0142x-42.285	0.3699
25-30	y = 7.9804x-40.523	0.3001
30-35	y = 7.9033x-40.495	0.2733
35-40	y = 8.1143x-41.086	0.2655
40-45	y = 7.9754x-41.253	0.2577
45-50	y = 7.8877x-41.421	0.247
50-55	y = 7.723x-41.533	0.2378
55-60	y = 7.3977x-41.457	0.2215
60-65	y = 7.2115x-41.761	0.1937
65-70	y = 7.3462x-42.678	0.1836
70-75	y = 7.4832x-43.647	0.1782
75-80	y = 8.2069x-45.714	0.2013
80-85	y = 8.8049x-47.849	0.2095
85-90	y = 9.3176x-49.781	0.2168
90-95	y = 10.213x-52.411	0.2372
95-100	y = 11.217x-55.268	0.2748

Appendix 9 Regression equations and R squared values for linear regressions of echo intensity (target strength) and log10 area of nocturnal moth wings from Chapter 4. All data were split into 5 kHz frequency bands between 20 and 100 kHz prior to running regressions.

Frequency Band (kHz)	Equation	R^2
20-25	y = 15.697x-44.685	0.5793
25-30	y = 14.283x-40.634	0.5217
30-35	y = 13.518x-38.58	0.4656
35-40	y = 13.211x-37.977	0.4204
40-45	y = 12.66x-36.908	0.3955
45-50	y = 12.168x-36.015	0.3893
50-55	y = 12.31x-36.817	0.3879
55-60	y = 12.065x-36.759	0.3821
60-65	y = 11.47x-36.106	0.3581
65-70	y = 11.452x-37.047	0.3377
70-75	y = 11.791x-38.957	0.3343
75-80	y = 11.936x-40.64	0.326
80-85	y = 12.231x-42.866	0.3119
85-90	y = 13.273x-46.756	0.3138
90-95	y = 13.791x-49.122	0.3157
95-100	y = 14.123x-50.985	0.3209

Appendix 10 Regression equations and R squared values for linear regressions of echo intensity (target strength) and log10 area of nocturnal moth bodies from Chapter 4. All data were split into 5 kHz frequency bands between 20 and 100 kHz prior to running regressions.

Frequency Band (kHz)	Equation	R^2
20-25	y = 8.1272x-40.859	0.209
25-30	y = 7.6591x-40.219	0.1679
30-35	y = 7.8447x-40.733	0.1591
35-40	y = 7.8179x-41.033	0.15
40-45	y = 8.446x-42.658	0.1651
45-50	y = 7.8286x-41.889	0.1413
50-55	y = 7.7233x-42.064	0.139
55-60	y = 7.0762x-41.318	0.1199
60-65	y = 6.6274x-41.112	0.097
65-70	y = 6.9276x-42.36	0.0947
70-75	y = 7.0464x-43.216	0.091
75-80	y = 7.7021x-45.126	0.1062
80-85	y = 8.009x-46.684	0.1081
85-90	y = 8.3791x-48.263	0.1115
90-95	y = 9.6078x-51.65	0.1306
95-100	y = 10.548x-54.38	0.1591